Volume 99 JULY-AUGUST 1956 Number 1

The Journal of Infectious Diseases

Founded 1904 by the John Rockefeller McCormick Memorial Institute for Infectious Diseases
under the Editorship of LUDVIG HEKTOEN and EDWIN O. JORDAN

WILLIAM H. TALIAFERRO, *Editor-in-Chief*

Advisory Editors

WILLIAM BURROWS · G. M. DACK · F. B. GORDON · R. W. HARRISON · STEWART A. KOSER
ROBERT M. LEWERT · KARL F. MEYER · C. PHILLIP MILLER · JAMES W. MOULDER
AARON NOVICK · F. G. NOVY · DAVID W. TALMAGE

PUBLISHED FOR THE JOHN ROCKEFELLER McCORMICK MEMORIAL FUND
OF THE UNIVERSITY OF CHICAGO

THE UNIVERSITY OF CHICAGO PRESS
CHICAGO · ILLINOIS · U.S.A.

The Journal of Infectious Diseases

The Journal of Infectious Diseases is devoted to the publication of original investigations dealing with the general phenomena, causation, and prevention of infectious diseases. It is the aim of the Journal to occupy a special field and to include only such contributions as bear with reasonable directness on the topics indicated in the title. The biology and chemistry of the various pathogenic organisms, the physiology and anatomy of the saprozoic processes that they initiate, and the hygienic and sanitary problems to which they give rise are considered to be especially within the scope of the undertaking.

This journal was established in connection with the John Rockefeller McCormick Memorial Institute for Infectious Diseases, [illegible] munificence of the late Mrs. Edith Rockefeller McCormick and the late Mr. Harold F. McCormick. Since 1941 it has been published in connection with the John Rockefeller McCormick Memorial Fund of the University of Chicago.

The Journal of Infectious Diseases is published bi-monthly by The University of Chicago Press, 5750 Ellis Avenue, Chicago, Illinois. The subscription price [illegible] year; $3.50 a volume; the price of single copies of current volumes is $1.00. ¶ Postage is prepaid by the publishers on all orders from the United States, Mexico, Cuba, Puerto Rico, Panama Canal Zone, Republic of Panama, Dominican Republic, Canary Islands, El Salvador, Argentina, Bolivia, Colombia, Chile, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, Peru, [illegible], Paraguay, Hawaiian Islands, Balearic Islands, Spain and Venezuela. ¶ Postage is [illegible] as follows: for Canada and Newfoundland, 40 cents on annual subscriptions (total $3.90); on single copies 10 cents (total $1.10); for all other countries in the Postal Union, $1 cents on annual subscriptions (total $3.75); on single copies 15 cents (total $1.15). ¶ Payment is requested [illegible] remittances payable to the University Press in postal or express money orders or bankdrafts.

The following are authorized agents:

For the British Empire, except [illegible] America, apply to The Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, England. Prices of yearly subscriptions and on single copies may be had on application.

Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

Business correspondence should be addressed to The University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

Communications for the editors and manuscripts should be addressed to Mrs. Dorothy W. Geyer, Assistant to the Editor, The Journal of Infectious Diseases, 5750 Ellis Avenue, Chicago 37, Illinois.

Entered as second-class matter August 1, [illegible] at Chicago, Illinois, under the Act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in the United States Postal Act of October 3, 1917, Section 1103, amended February 28, 1925, authorized [illegible], 1918.

Notice to Subscribers: If you change your address, please notify us and your local postmaster immediately. The Post Office does not forward second-class mail.

J. Infect. Dis. Vol. 99, No. 1

QUANTITATIVE STUDIES OF THE COLLAGENASE-LIKE ENZYMES OF CERCARIAE OF *SCHISTOSOMA MANSONI* AND THE LARVAE OF *STRONGYLOIDES RATTI*

ROBERT M. LEWERT AND CHANG-LING LEE

From the Department of Microbiology, The University of Chicago, Chicago 37, Illinois

During passage through the skin of the vertebrate host, the infective larvae of *Schistosoma mansoni*, *Strongyloides ratti* and certain other helminths alter the polysaccharide-containing protein of the basement membrane and ground substance of the dermis. These alterations of host glycoprotein have been revealed by histochemical methods and are attributed to enzymatic activity of the secretions of the infective larvae. Furthermore, qualitative studies of the secretions and extracts of the skin penetrating larvae show that there is a correlation between the presence of a collagenase-like enzymatic activity and the ability to cause changes in the host glycoprotein.[1] The present study is a continuation of the investigation of the enzymes of these helminth larvae in order to characterize their activity more fully and to investigate the effects of various substances on these enzyme systems. This has been done in part by comparision with the activities of bacterial collagenase from toxin of *Clostridium welchii* type A and with those of trypsin, using quantitative methods and a variety of substrates. The methods include the determination of dye release from azo dye-coupled collagens, the hydroxyproline release from collagen substrates and the tyrosine release from a hemoglobin substrate. In addition, where warranted, in vivo tests have been performed to determine whether in vitro enzyme inhibitors affect penetration of the skin by the living larvae.

Received for publication September 6, 1955.

These investigations were conducted under the sponsorship of the Commission on Parasitic Diseases, Armed Forces Epidemiological Board, and were supported in part by the Office of the Surgeon General, Department of the Army, and by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago.

MATERIALS AND TECHNIQUES

Saline extracts of cercariae of *Schistosoma mansoni* and of filariform larvae of *Strongyloides ratti*, *Clostridium welchii* toxin,* and crystalline trypsin† were compared in most of the experiments. Living larvae of both helminths as well as living larvae and extracts of larvae of *Ancylostoma caninum*, and *Nippostrongylus muris* were also compared in a similar manner. The procedures used in the concentration of the nematode filariform larvae and in preparing extracts have been reported previously.[1] Since large numbers of cercariae of *S. mansoni* were required, efficient methods of maintenance of this infection in the laboratory were devised and are presented in an accompanying report.[2] To remove any suspended debris, cercariae were decanted through a 100 mesh screen into one liter pharmaceutical graduates. These cercarial suspensions were concentrated by chilling at 5 C for 2 to 16 hours. The subsequent washing, lyophilization, and preparation of extracts were identical to those used for nematode larvae.[1] In some cases the residue from the first extraction was extracted a second time by homogenizing in saline and stored overnight at 5 C. The activity of the second extract was usually less than 50% that of the first but was high enough to be used without further concentration.

* We are indebted to Dr. William Burrows for this preparation, a lyophilized toxin containing approximately 90% by weight lactose and lacking in theta toxin.

† Worthington Biochemical Corp., 2× crystallized.

1. Lewert, R. M. and Lee, C. L. 1954, Studies on the passage of helminth larvae through host tissues. I. Histochemical studies on extracellular changes caused by penetrating larvae. II. Enzymatic activity of larvae in vitro and in vivo. J. Infect. Dis. 95: 13–51.
2. Lee, C. L. and Lewert, R. M. 1956, The maintenance of *Schistosoma mansoni* in the laboratory. J. Infect. Dis. 99: 15–20.

The dry weight of larvae was computed after lyophilizing at −30 C in the presence of phosphorus pentoxide in vacuum. Each milligram thus prepared contained approximately 12,500 *S. mansoni*, 40,000 *S. ratti*, 17,000 *N. muris*, or 2,500 *A. caninum* larvae.

The substrates used included hide powder, beef tracheal cartilage, native bovine collagen,* and hemoglobin. Azocollagen (azocoll) and azocartilage were prepared from the first two substances using the technique described by Oakley et al,[4] the only modification being the subdivision of the resulting colored substrate by grinding in anhydrous acetone in a ball mill for 72 hours. After drying only the material passing an 80-mesh screen was used.

The release to solution of the azo dye from the coupled substrates was determined quantitatively. Optimal conditions for the system were determined using various buffers, salt concentrations, amounts of enzyme, etc. as described in results. With the exception of a few specially designed experiments, tests were conducted as follows: 5 mg of substrate were incubated with an aliquot of enzyme in a 10×75 mm pyrex tube at 37 C for 120 minutes. A total volume of 1 ml buffered with 0.1 *M* phosphate at pH 7.4 was used unless otherwise indicated. Tests were run in triplicate or quadruplicate depending on the amount of helminth extract available. Tubes were tightly stoppered, fixed to the horizontal table of a rocking apparatus and the mixture gently rocked 12 times per minute throughout the incubation period. After incubation all tubes were inactivated by heating at 60 C for 10 minutes and centrifuged. In a few of the earlier experiments in this series (results, fig. 1 and fig. 4), dye release was measured in a Klett-Summerson colorimeter. In all other experiments 0.5 ml of the supernatant from each tube was transferred to a silica micro cell and the optical density was read at 540 mμ with a Beckman spectrophotometer. Under the conditions described, saline extracts from 0.2 mg of *S. mansoni* cercariae, 0.5 mg of *S. ratti* larvae, 0.25 mg of *Cl. welchii* toxin or 0.0001 mg of trypsin resulted in approximately equivalent dye-release from azocoll and were used in most of the experiments. As might be expected, helminth extracts prepared at different times varied slightly in their activity. This depended in part on original dry weight per unit volume of extracting solution and in part on the length of time and how the material was stored. For example, the activity of the extract of *S. ratti* decreased with the length of time larvae were stored at −30 C before lyophilization. The activity of lyophilized larvae, cercariae, and toxin did not decrease when stored in a dessicator at 4 C and extracts were stored at −30 C for more than a year without appreciable loss of activity. To eliminate the possibility of activity due to the presence of contaminating bacteria, the activity of Seitz filtered extracts and extracts containing penicillin and streptomycin were tested. No activity attributable to bacterial contaminants was found in the systems tested.

Various ions, usually as sulfates and selected from substances known to have activating or inhibiting effects on various enzyme systems, were added to the system in trace amounts or in concentrations up to 10^{-3} *M*. These included As^{+++}, Ca^{++}, Co^{++}, Cu^{++}, CN^{-}, Fe^{+++}, Fe^{++}, Mg^{++}, Mn^{++}, Hg^{++}, Ni^{++}, Pb^{++}, and Zn^{++}. Other substances such as chelating agents, sulfhydryl-containing or S-H-binding agents, oxidizing and reducing agents were tested in a similar manner at concentrations indicated in the results. These included the disodium salt of ethylenediamine tetra-acetic acid (EDTA), N-ethyl malemide (NEM), sodium iodoacetate, l-cysteine HCl, ascorbic acid, dehydroascorbic acid, benzoquinone, alkaline quinone and hydroquinone. The trypsin inhibitors, ovomucoid and soy bean trypsin inhibitor, were utilized in a number of experiments. In each instance the system was buffered as described above. The results are for convenience expressed as per cent dye release, as compared to that released in the standard enzyme or helminth extract plus substrate. A figure greater or less than 100% represents activation or inhibition of the system, respectively.

Hydroxyproline determinations were made using the technique of Neuman and Logan[5] on 10 to 30 mg of washed substrate. The tests were carried out by incubating the substrate with enzyme at 37 C for 20 or 46 hours with continuous agitation in 0.1 *M* phosphate buffer at pH 7.0 with a volume of 1 ml per 10 mg of substrate. One mg of streptomycin and 500 units of penicillin per ml were included in each tube. After incu-

* Kindly supplied by Dr. A. Veis, Armour Research Laboratories, see Veis and Cohen.[3]

3. Veis, A. and Cohen, J. 1954, The degradation of collagen. A method for the characterization of native collagen. J. Am. Chem. Soc. **76**: 2476–2478.

4. Oakley, C. L., Warrack, G. H. and VanHeyningen, W. E. 1946, The collagenase (α toxin) of *Cl. welchii* type A. J. Path. & Bact. **58**: 229–253.

5. Neuman, R. E. and Logan, M. A. 1950, The determination of hydroxyproline. J. Biol. Chem. **184**: 299–306.

bation the tubes were centrifuged at 2000 RCF for 5 minutes and samples of the supernatant and of the precipitate were subjected to hydrolysis with 1 ml of 6 *N* HCl at 125 C and 15 lbs. pressure for 3 hours. Longer periods of hydrolysis did not increase the release of hydroxyproline. The hydrolysates were neutralized with 2.5 *N* NaOH. Hydroxyproline determinations were then made on 1 ml of the original supernatant, neutralized hydrolyzed supernatant, and neutralized hydrolyzed precipitate. Hydroxyproline controls were prepared as were the usual buffer controls. The effects of EDTA, NEM, cysteine, and ovomucoid on this system were also studied.

Hemoglobin digestion was measured as tyrosine released using a modified Anson technique[6] in which the test was run at one tenth the standard volume to conserve the helminth extracts and increase the sensitivity.

Two methods were used to test the effect enzyme inhibitors might have on the skin-penetrating ability of the helminth larvae. First, rats approximately 150 g in weight were anaesthetized with nembutal and their abdominal skin shaved and washed. Four layers of surgical gauze 1.5 cm square were applied to the areas to be tested. The rats were then injected intravenously with 1.5 ml of a 1% solution of Evans blue in saline and equal numbers of larvae in suspending solution were applied to each gauze square. The inhibitor or substance to be tested was added to the cercarial or larval suspension immediately prior to adding them to the gauze square. At the end of 15 minutes the gauze was removed and the blue spots in the exposed areas counted and compared with the control area. Reduction in number or absence of the blue spots indicated inhibition of penetration. Secondly, mice were exposed by tail immersion to 150 cercariae of *S. mansoni* which were suspended in water or in a solution of the inhibitor being tested. The number of adult worms present was counted 6 weeks later when they could readily be recovered by perfusion techniques.

6. Northrop, J. H., Kunitz, M. and Herriott, R. M. 1948, Crystalline Enzymes, ed. 2, Columbia University Press.

RESULTS

Activity relative to number and dry weight of larvae.—We have reported previously that living cercariae of *S. mansoni* or living filariform larvae of *S. ratti* when incubated with azocoll cause dye to be released from this substrate.[1] In the present quantitative study, when increasing increments of living larvae of each of these species are used the amount of dye released was proportionately increased as is shown in figure 1. Cercariae of *S. mansoni* in increasing amounts up to 8000 per ml, increasing amounts of *Cl. welchii* toxin, and larvae of *S. ratti* up to 80,000 per ml resulted in dye release that is plotted essentially as a straight line. The decrease in the readings of the *S. ratti* series at 90,000 to 100,000 per ml is accounted for by

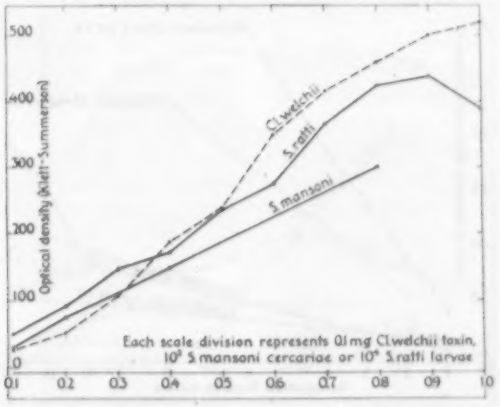


FIG. 1.—Activity of living larvae on azocoll substrate. Five mg azocoll in 1 ml *M*/10 phosphate buffer at pH 7.0, incubated 4 hours at 37 C.

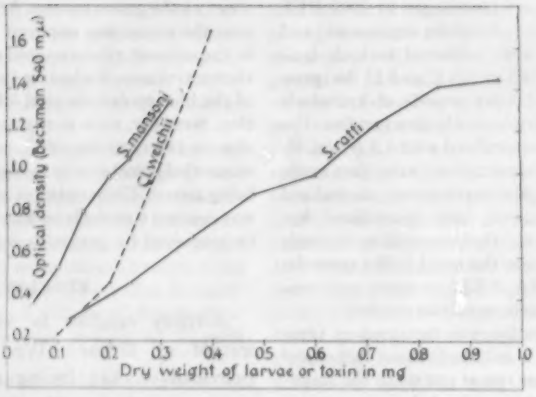


FIG. 2.—Activity of saline extracts* of helminth larvae on azocoll. Five mg azocoll in 1 ml *M*/10 phosphate buffer at pH 7.4, incubated 2 hours at 37 C.

* 0.1 mg of *S. mansoni* represents the dry weight of approximately 1250 cercariae.
0.1 mg of *S. ratti* represents the dry weight of approximately 4000 filariform larvae.

the death of large numbers of the larvae in the system early in the incubation period. Similarly, increasing amounts of extracts of these larvae, measured as dry weight before extraction, also released dye from the substrate in direct relation to the amount present (fig. 2).

Activity relative to duration of incubation.—With large numbers of living larvae or with large amounts of extract, dye release from 5 mg of azocoll in a total volume of 1 ml was perceptible to the eye in 15 minutes or less. With a small amount of extract of *S. mansoni* (0.1 mg) or a similar amount of bacterial toxin, dye release increased in a linear fashion with time of incubation. If larger amounts of each were used, the slope of this line was greatly increased with most of the dye being released from the substrate within 12 hours. The amount released in a definite period was of course affected by the degree of

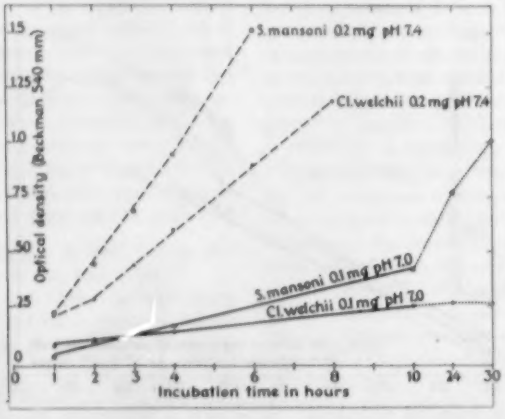

Optical density (Beckman 540 mm)
S. mansoni 0.2 mg pH 7.4
Cl. welchii 0.2 mg pH 7.4
S. mansoni 0.1 mg pH 7.0
Cl. welchii 0.1 mg pH 7.0
Incubation time in hours

FIG. 3.—Activity of extract of cercariae of *S. mansoni* on azocoll with increasing time as compared to the activity of *Cl. welchii* toxin. Five mg azocoll in 1 ml of *M*/10 phosphate buffer at 37 C.

labeling of the azocoll as well as by the pH and the activity of the individual batch of larval extract. The results of one such experiment are shown in figure 3. In all later experiments dealing with inhibition or activation, the amount of extract, toxin, or trypsin was so adjusted that at 2 hours incubation at 37 C an optical density of 0.5 to 0.6 resulted after subtracting the buffer control.

Heat sensitivity.—The helminth extracts as well as *Cl. welchii* toxin and trypsin were heat sensitive with 50% of the activity of *S. mansoni* extract, 80% of the activity of *S. ratti* extract, 60% of the tryptic activity, and 95% of the activity of the *Cl. welchii* toxin on the azocoll destroyed by heating to 56 C for 30 minutes. Heating at 60 C for 10 minutes destroyed 90–100% of the activity of all four preparations.

Effect of buffer.—The effect of pH on activity in *M*/10 phosphate buffer was determined in a number of experiments, one of which is illustrated in figure 4. The optimal activity of the extract of cercariae of *S. mansoni* was near pH 7.5, of *S. ratti* extract near pH 7.0, while the *Cl. welchii* toxin was almost uniformly active against azocoll over the range of pH 6.0 to 7.5. Extracts of two other skin-penetrating larvae were also tested at varying pH. Equivocal results had been obtained previously with extracts of the filariform larvae of *Ancylostoma caninum* and some activity was demonstrated by living larvae of this species.[1] No activity could be found at this time over the pH range tested nor was any found in extracts of the filariform larvae of *Nippostrongylus muris*. Trypsin, not shown on this figure, exhibited its expected activity with a maximum at pH 7.4 in this system. Phosphate buffer at less than *M*/10 final concentration in the system at 2 hours incubation gave progressively lower dye release as the molarity was decreased, with *M*/50 buffer giving approximately 50% that of the *M*/10. Buffers other than the Sorenson phosphate buffer were tested and included Michaelis' veronal, McIlvaine-Lillie citric acid, glycyl glycine, and Gomori's trihydroxy-methyl amino methane. All were less satisfactory than the phosphate buffer with glycyl glycine buffer being least satisfactory. After desalting in an electric desalter for 5 to 15

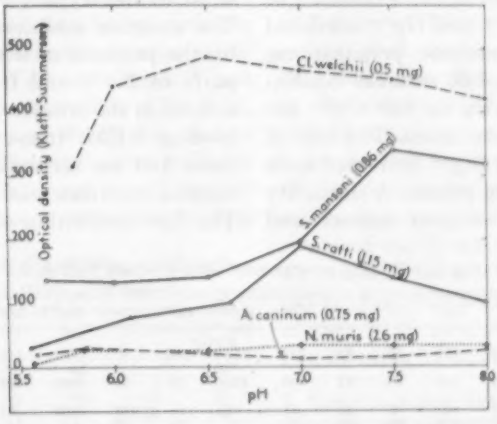


FIG. 4.—The effect of pH on dye release from azocoll. Five mg azocoll in 1 ml *M*/10 phosphate buffer, incubated 2 hours at 37 C.

minutes, all enzyme preparations were inactivated with a recovery of only the *Cl. welchii* toxin to 70% of former activity when buffer was replaced. During desalting, some foaming was noted in all preparations.

Effect of trypsin inhibitors.—In the presence of sufficient ovomucoid or soy bean trypsin inhibitor to completely inhibit the amount of trypsin releasing equivalent amounts of dye, no inhibition of *S. mansoni* or *S. ratti* extract or *Cl. welchii* toxin resulted. The ability of 10^{-4} mg of trypsin to release dye from the substrate was 90% inhibited by 10^{-4} mg of ovomucoid and completely inhibited by 10^{-3} mg of ovomucoid or 10^{-4} mg of soy bean trypsin inhibitor. Neither of these substances when present in as much as 100 times this concentration had any inhibitory effect on extracts of *S. mansoni*, *S. ratti*, or *Cl. welchii* toxin, and in fact, uniformly slightly activated each.

Effect of various ions.—The effects of various ions at a concentration of $10^{-4}M$ on the helminth extracts and enzymes tested are summarized by table 1. Their effects appear somewhat erratic but reveal certain points of similarity and difference in the substances tested. The metals Co^{++}, Cu^{++}, and Hg^{++} inhibited all four of the enzyme preparations, with the extract of *S. mansoni* exhibiting more sensitivity to the Cu^{++} ion than the other substances. The ions of As^{+++}, Ca^{++}, and Mg^{++} activated each enzyme to varying extent. A similarity between the *S. mansoni* extract and trypsin is noted in the fact that both were partially inhibited by Zn^{++} while the remaining substances tested were essentially unaffected. These two and the *S. ratti* extract were also inhibited by divalent iron. It is interesting to note that only the *S. ratti* extract was inhibited by the presence of Ni^{++} and that only the *S. mansoni* extract exhibited inhibition in the presence of CN^{-}.

The effects of S-H-containing compounds and S-H-binding substances.—Preliminary studies indicated that the enzymes released by the living larvae of *S. mansoni* and *S. ratti*, as well as the toxin of *Cl. welchii*, were strongly inhibited by the presence of small amounts of S-H-containing compounds such as cysteine, glutathione and BAL. Trypsin activity was not reduced. These preliminary results were followed by a quantitative study of the effect of cysteine on the ability of helminth extracts, toxin, and trypsin to release dye from the azocoll substrate. From the results presented in table 2, it is seen that the extract of *S. ratti* closely resembled the *Cl. welchii* toxin in being inhibited by the concentration of cysteine used while the extracts of *S. mansoni* and trypsin were not inhibited. The cysteine inhibition was abolished by the presence of less than equimolar parts of Zn^{++}, and inhibition was decreased in the presence of the sulfhydryl binding NEM. Iron-cysteine combinations had no activating effect on the various enzymes and extracts tested. The Zn^{++} inhibition of *S. mansoni* and

TABLE 1.—*Effect of various ions* on dye release from azocoll.*

	Buffer	As^{+++}	Ca^{++}	Co^{++}	Cu^{++}	CN^{-}	Fe^{+++}	Fe^{++}	Pb^{++}	Mg^{++}	Mn^{++}	Hg^{++}	Ni^{++}	Zn^{++}
Cl. welchii toxin	100	105	145	73	57	118	113	105	97	116	117	21	100	100
S. ratti extract	100	129	145	90	72	108	122	66	89	104	123	52	61	96
S. mansoni extract	100	119	142	75	43	88	95	76	101	132	92	40	91	81
Trypsin	100	142	128	66	62	137	135	71	103	121	141	26	97	88

5 mg azocoll, 1 ml *M*/10 phosphate buffer pH 7.4 incubated 2 hours at 37 C.
* 10^{-4} *M*.

TABLE 2.—*Effect of cysteine on dye release from azocoll.*

	Buffer	Cysteine				NEM*	Zn^{++}	Fe^{+++}			
		2×10^{-3} *M* (1)	10^{-3} *M*	10^{-3} *M*	10^{-4} *M* (2)	2×10^{-3} *M* (3)	10^{-3} *M* (4)	10^{-4} *M* (5)	(1+3)	(1+4)	(2+5)
Cl. welchii toxin	100	39	35	100	109	24	93	113	76	100	96
S. ratti extract	100	6	8	35	107	110	104	122	91	100	100
S. mansoni extract	100	93	99	103	105	16	79	95	66	117	100
Trypsin	100	103	108	100	92	94	85	135	90	135	115

5 mg azocoll in 1 ml *M*/10 phosphate buffer pH 7.4 incubated 2 hours at 37 C.
* N-ethyl-maleimide.

trypsin was lost in the presence of cysteine. The sulfhydryl-binding agent, NEM, was inhibitory to only the *Cl. welchii* toxin and the *S. mansoni* extract. The latter inhibition was only partly reversed by the presence of equimolar concentrations of cysteine. Relatively high concentrations of sodium iodoacetate (*M*/20) resulted in only 45% inhibiton of the *S. mansoni* extract and slight (14%) inhibition of the *Cl. welchii* toxin while trypsin was not affected and *S. ratti* not tested.

The effect of a chelating agent.—As the inactivation of the enzymes by sulfhydryl containing compounds might be a phenomenon associated with their ability to bind metal ions essential to the activity of the enzyme, the effect of a chelating agent on the various systems was studied. In table 3 the results of one such experiment with the disodium salt of ethylenediamine tetraacetic acid (EDTA) are shown. When present at a concentration of 10^{-3} *M*, this substance completely or almost completely inhibited the activity of both the *S. ratti* extract and the *Cl. welchii* toxin on the azocoll substrate. It was without effect on either the *S. mansoni* extract or trypsin. As can be seen from the table, the inhibitory effects were nullified by the presence of equimolar parts of Zn^{++}. In other experiments, inhibition of *Cl. welchii* toxin and of the extract of *S. ratti* by EDTA to a lesser degree was noted even at a concentration of 10^{-4} *M*. In spite of its lack of effect on the extract of *S. mansoni*, like the S-H-containing compounds, EDTA at 10^{-3} *M* inhibited the activity of living *S. mansoni* cercariae on the azocoll substrate.

TABLE 3.—*Effect of a chelating agent* and Zn^{++} on dye release from azocoll.*

	Buffer	Zn^{++}†	EDTA*	Zn^{++} and EDTA
Cl welchii toxin	100	98	7	102
S. ratti extract	100	98	0	120
S. mansoni extract	100	79	115	82
Trypsin	100	85	110	120

* EDTA = Disodium salt of ethylene-diamine tetra-acetic acid at 10^{-3} *M*.
† At 10^{-3} *M*.

Effects of various reducing and oxidizing agents.—Some substances were tested because of their ability to inhibit "mucase" enzymes[7] or because of their reducing activity. Inhibition of activity by ascorbic acid did not parallel that of reducing agents such as cysteine. It had an inhibitory effect only on the extract of *S. mansoni* (50%) and trypsin (15%) and then only when present in relatively high (*M*/50) concentration. Dehydroascorbic acid at the same concentration had essentially the same effect. Benzoquinone at relatively low concentration (3×10^{-4} *M*) partially inhibited the *S. mansoni* extract (40%), *Cl. welchii* toxin (30%) and trypsin (40%) but was without effect on the extract of *S. ratti*. Alkalinized quinone lost its capacity to inhibit except for the

7. Roseman, S. and Dorfman, A. 1952, Effect of gentisic acid and related compounds on bovine testicular hyaluronidase. J. Biol. Chem. **199**: 345–355.

Cl. welchii toxin (30%) while hydroquinone gave about the same general low level of inhibition (10–20%) of all enzymes tested.

Activity against azocartilage.—When amounts of trypsin, *Cl. welchii* toxin, *S. mansoni* extract, and *S. ratti* extract that give equivalent dye release from azocoll were incubated with 5 mg of azocartilage, the dye release was not equivalent. The extract of *S. mansoni* was most active, that of *S. ratti* slightly more than half as active as the *S. mansoni*, and the *Cl. welchii* toxin slightly less than half as active as *S. mansoni*. Trypsin was totally without activity against this substrate preparation. Both EDTA and cysteine which inhibited dye release from azocoll also inhibited the dye release from the azocartilage system.

Release of hydroxyproline from substrates.—Preliminary tests of the activity of the various helminth extracts and bacterial toxin against hide powder were made using descending paper chromatograms, which revealed that proline or hydroxyproline was released. A limited number of quantitative determinations of hydroxyproline release from hide powder and tracheal cartilage were performed and the data are presented in table 4. Since 6 to 15 times as much extract were required in each test because the test is relatively insensitive as compared to the azocoll method, the amount of data that could economically be acquired was limited. All of the substances acting on the azocoll substrate, trypsin, *Cl. welchii* toxin, *S. mansoni* extract, and *S. ratti* extract, also released hydroxyproline from hide powder. In each, 6 to 10% of that released was found as free amino acid while the greater portion, roughly 90%, was present in the polypeptide fraction. No direct comparison of the completeness of digestion of the substrate was made. It may be pointed out that 3×10^{-4} mg of trypsin in this system digested the same amount of substrate (60%) as did 3 mg of *S. mansoni* larvae measured as dry weight before extraction. Equivalent dye release from azocoll was obtained from $\frac{1}{3}$ of this quantity of trypsin and 1/15 of this quantity of *S. mansoni* extract. It is interesting that the extract of *A. caninum*, which exhibited little or no activity against azocoll, also had only a slight ability to release hydroxyproline from the uncoupled hide powder and that the extracts of *N. muris* that were negative to azocoll were also without activity here. The ability to free hydroxyproline from cartilage also paralleled activity on the azocartilage substrate in that trypsin was essentially inactive while *S. mansoni*, *S. ratti*, and *Cl. welchii* toxin all released hydroxyproline from this substrate. Again about 10% of that released was found as free amino acid.

In general the substances inhibitory to dye release from azocoll and azocartilage also inhibited hydroxyproline release from the uncoupled substrates.

TABLE 4.—*Percent hydroxyproline released from hide powder or cartilage substrate.*

Substrates (30 mg) Enzymes*	Hide powder in:			Cartilage in:		
	Free	Hydrolysate	Total	Free	Hydrolysate	Total
C. welchii toxin	7.0	68.2	75.2	5.8	42.3	48.1
S. mansoni extract	4.2	55	59.2	8.4	61.5	69.9
S. ratti extract	2.8	24.3	27.1	4.4	40.3	44.7
A. caninum extract	0.9	3.9	4.8	1.1	0	1.1
N. muris extract	0	0	0	0	0	0
Trypsin	4.0	50.5	60.5	1.6	0	1.6

Total volume 3 ml of pH 7.0, *M*/10 phosphate buffer, penicillin and dihydrostreptomycin added, incubated at 37 C for 20 hours.

* All enzymes 3 mg except trypsin 3×10^{-4} mg.

TABLE 5.—*Effect of inhibitors on the release of hydroxyproline from collagen by various enzymes.*

	Cl. welchii toxin	*S. mansoni* extract	*S. ratti* extract	Trypsin 2×10^{-3} mg
I. Cartilage				
Phosphate buffer (PB) pH 7.0, 0.1 *M*	100	100	100	—
EDTA* 2×10^{-3} *M* in PB	35	107	14	—
NEM† 2×10^{-3} *M* in PB	71	41	56	—
L-cysteine HCl 2×10^{-3} *M* in PB	111	94	78	—
II. Hide powder				
Phosphate buffer pH 7.0, 0.1 *M*	100	100	100	100
Ovomucoid 10^{-3} mg	92	111	95	1

Substrate 20 mg, enzymes 2 mg in 2 ml of pH 7.0, *M*/10 phosphate buffer incubated at 37 C for 20 hours with continuous mixing, penicillin and dihydrostreptomycin added, expressed in percent of substrate hydroxyproline released.
* Ethylene-diamine tetra-acetic acid disodium salt.
† N-ethyl-maleimide.

This is illustrated by table 5, where it may be seen that EDTA inhibited the activity of *Cl. welchii* toxin and *S. ratti* extract but had no effect on the *S. mansoni* extract. N-ethyl maleimide inhibited *S. mansoni* extract and the *Cl. welchii* toxin and with this system also inhibited the activity of the *S. ratti* extract. The *S. ratti* extract, as in previous experiments, was most sensitive to the presence of cysteine while in this experiment no inhibition of *Cl. welchii* toxin was apparent in the presence of the amount of cysteine used. The amount of cysteine present was only ¼ that necessary to give 50% inhibition of toxin in the azocoll system. The trypsin inhibitor, ovomucoid, inhibited only the activity of trypsin and, as in the azo-dye systems, had no perceptible effect on the bacterial toxin or the helminth extracts.

The effect of the various enzymes on a reconstituted "native" collagen were not as marked as that found in the previous substrates. Relatively slight activity was evidenced by the *S. mansoni* extract and the *S. ratti* extract, which released about 5% of the hydroxyproline present in 20 hours incubation. Only the *Cl. welchii* toxin exhibited activity of a higher level and this released slightly more than 8% in 20 hours. Even at 46 hours of incubation this did not approach the hydroxyproline release found for the other substrates, as the helminth extracts liberated only 5–6% while the bacterial toxin liberated almost 40% of that present in the substrate. Trypsin was essentially inactive as were extracts of *N. muris* and *A. caninum*.

Tyrosine release from hemoglobin.—In a previous report[1] no degradation of urea-denatured hemoglobin could be shown in various of the nematode extracts tested, including that of *Strongyloides*. Sufficient material of *S. mansoni* was not available at the time to carry out conclusive tests. More recently, it has been possible to compare the ability of *S. mansoni* extract, *Cl. welchii* toxin and trypsin to release tyrosine from a denatured hemoglobin substrate. The results of one experiment shown in table 6 indicate that the toxin of *Cl. welchii* was inactive and the extract of *S. mansoni* had the ability to release tyrosine from the substrate. Although trypsin was inhibited in the system by the presence of ovomucoid, the extract of *S. mansoni* was unaffected.

Additional histochemical studies.—Our previous investigations have shown that during skin penetration by helminths there were extensive changes in the dermal connective tissue. These were primarily noted in the polysaccharide-containing protein of the basement

TABLE 6.—*Tyrosine release from hemoglobin.*

	Tyrosine released* (micrograms)
S. mansoni extract, 0.5 mg	56
S. mansoni extract, 0.5 mg+ovomucoid 10^{-3} mg	58
Trypsin, 2×10^{-3} mg	56
Trypsin, 2×10^{-3} mg+ovomucoid 10^{-3} mg	<5
C. welchii toxin, 2.0 mg	0

11 mg hemoglobin in 1 ml incubated 60 minutes at 37 C.
* Average of three determinations.

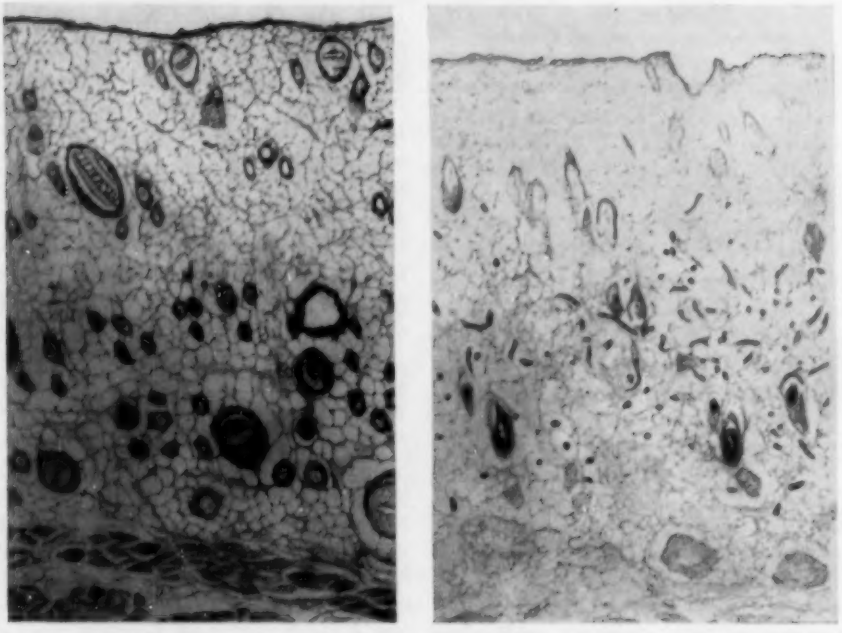


FIG. 5.—Reduction in protein-bound sulfhydryl groups in rat skin following exposure to larvae of *S. ratti*.

A. (Left side) Normal skin, 10 μ section, Barrnett method.

B. (Right side) Adjacent area of skin, 10 μ section, exposed 30 minutes to several thousand filariform larvae of *S. ratti*. Note the over-all reduction in staining. Sections of larvae are numerous in the dermis.

membrane and ground substance. That collagen and reticulin were also affected has been shown by using techniques to demonstrate argyrophilic material. With silver stains for reticulum, the reticular fibers in the dermis were altered as the skin was penetrated by the cercariae of *S. mansoni* and they disappeared in the immediate vicinity of the larvae or else lost their argyrophilia. Collagen fibers were also altered in the immediate vicinity of the larvae in the dermis in that they became argyrophilic. An additional change was noted after massive exposure of the skin to larvae of either *S. mansoni* or *S. ratti*. Throughout the dermis, as illustrated in figure 5, there was a general decrease in the number of protein-bound sulfhydryl groups that could be demonstrated by the technique of Barrnett.[8]

In vivo studies.—The substances which inhibited the enzymes of skin-penetrating larvae in vitro were tested for their effect on the ability of these larvae to penetrate the skin of rats and mice. Many of the substances tested, e.g., Cu^{++} or Hg^{++}, when used in concentration great enough to inhibit penetration were also lethal to the organisms or resulted in an obvious loss of motility and impairment of normal activities. Those which were not obviously detrimental to normal activity of the larvae were further investigated and included NEM at 10^{-7} *M*, EDTA at 10^{-3} and 10^{-4} *M*, and ascorbic acid at 10^{-3} *M*. Of these only EDTA gave

8. Barrnett, R. J. 1953, The histochemical distribution of protein-bound sulfhydryl groups. J. Nat. Cancer Inst. **13**: 905–925.

TABLE 7.—*The effect of EDTA* and NEM† on skin penetration by* S. mansoni *cercariae.*

Group	I	II	III	IV
Media	Dechlorinated water	Phosphate buffer 10^{-4} *M* pH 7.0	EDTA 10^{-2} *M* in water	NEM in water
Worms recovered	45 ± 10.3	47.8 ± 6.42	16.4 ± 2.64	54.4 ± 6.17

8 female CF 1 mice per group, each exposed to 150 cercariae by tail
* Ethylene diamine tetra-acetic acid disodium salt.
† N-ethyl-maleimide.

apparent inhibition as judged by the Evans blue response.[1] It completely inhibited the development of Evans blue at the area of the skin exposed to *S. ratti* and was associated with fewer spots at the area exposed to *S. mansoni* than in control areas exposed to cercariae without EDTA. With the latter species there was a statistically significant decrease in the number of worms developing in the exposed mice as shown in table 7. It is to be noted in the same table that NEM at 10^{-7} *M* is without effect.

DISCUSSION

We have suggested in a previous report that the activity of some helminth-produced enzymes, liberated as the infective larvae penetrate the skin of the mammalian host, are collagenase-like and share certain features with the toxin of *Cl. welchii*. The present study further characterizes the proteolytic activities of the enzyme or enzymes present in the cercariae of *S. mansoni* and the filariform larvae of *S. ratti* and compares these with bacterial collogenase and trypsin. The presence of proteolytic enzymes in these larvae was first shown in histochemical and in vivo studies which revealed that the living larvae, as they penetrate the skin, alter polysaccharide-containing protein, collagen fibers, reticular fibers, and protein-bound sulfhydryl groups. Furthermore, the living larvae in vitro have been shown to act on azo dye-coupled collagens and gelatin. Sterile saline extracts of these larvae exhibit a high degree of activity and are capable of degrading azo dye-coupled collagens, uncoupled collagens, gelatin, frozen dried skin sections and, in one case, hemoglobin. Although there are many points of similarity, it is apparent that the larvae of *S. mansoni* and *S. ratti* possess proteolytic "penetration" enzymes that differ not only from each other but also differ from trypsin and bacterial collagenase, with which they have been compared. A summary of some of their pertinent characteristics is made in table 8.

The helminth enzymes and bacterial collagenase have been compared to trypsin primarily because this is active in the same range of pH and is available as a highly purified preparation with relatively well-defined characteristics. Trypsin differs markedly from the

TABLE 8.—*Partial summary of activities of helminth extracts, bacterial toxin and trypsin.*

	S. mansoni extract	*S. ratti* extract	*Cl. welchii* toxin	Trypsin
Hide powder, azocoll, gelatin	+	+	+	+
Cartilage and azocartilage	+	+	+	–
Reconstituted native collagen	±	±	+	±
Bovine hemoglobin	+		–	+
Trypsin inhibitors	N	N	N	I
Cysteine or EDTA*	N	I	I	N
NEM† or iodoacetate	I	N	I	N
Ascorbic acid or dehydroascorbic acid	I	N	N	I
Benzoquinone	I	N	I	I
Alkaline quinone	N	N	I	N
Cu^{++} Hg^{++} Co^{++}	I	I	I	I
Mg^{++} Ca^{++}	A	A	A	A
Optimal pH	7.5	7.0	6–8	7.4

* Ethylenediamine tetra acetic acid disodium salt.
† N-ethyl-maleimide.
+, activity; –, no activity, ±, equivocal or slight.
I, inhibition; N, not affected, A, activation.

collagenases tested in being without activity against cartilage or azocartilage although it is active on hide powder and azocoll. Its activity on all substrates tested can be completely inhibited by ovomucoid or soy bean inhibitor, substances which are without inhibitory effect on the helminth enzymes or bacterial toxin. A further point of difference between trypsin and other enzymes is the fact that dilute normal serum (1:1000) strongly inhibits trypsin but is without effect on the helminth extracts and activates the *Cl. welchii* toxin. Data for effects of serum on collagenase have not been presented in the section on results as a more complete study will be part of a future report.

The comparison of the helminth enzymes with bacterial collagenase was originally made because of the similarity of action of collagenases and penetrating larvae on the acellular glycoprotein elements of connective tissue as shown by histochemical methods. The present investigation has shown that these helminth enzymes are active on a variety of collagen-containing substrates but their activity against a purified native collagen is slight in comparison to the activity of the *Cl. welchii* toxin. For this reason we prefer to term them collagenase-like, even though they share many characteristics of the bacterial collagenase.

The use of hide powder or other altered collagens as a substrate is not an exclusive test for the presence of collagenase activity as it has been shown that altered collagens such as azocoll may be solubilized by a variety of proteolytic enzymes.[9] However, as we and others[4] have shown, the dye release from azocoll can be used as an extremely sensitive, reproducible, quantitative test. Thus it is extremely valuable to give a baseline for the quantitative comparison of proteolysis when only minute amounts of the substance to be tested are available. The addition to this system of inhibitors of specific enzymes makes possible the elimination of non-collagenase types of proteolysis such as that of trypsin. The use of an azocartilage substrate complements the tests above as it is not solubilized by tryptic action but is acted upon by collagenase.

The enzyme present in extracts of cercariae of *S. mansoni* resembles bacterial collagenase and differs from trypsin in its ability to act on cartilage in vitro and on native polysaccharide-containing protein in vivo. Like collagenase it is unaffected or slightly activated by specific trypsin inhibitors but is inhibited by sulfhydryl-binding agents such as NEM, iodoacetate, and traces of heavy metals. These findings suggest that both contain sulfhydryl groups which must be uncombined to have active enzymes. The cercarial enzyme differs from the toxin preparation in having a more specific pH optimum in the system tested, in being sensitive to ascorbic acid, and in its ability to digest hemoglobin. Other preparations of *Cl. welchii* toxin are capable of digesting hemoglobin[10] but the one used in this study did not. The extract of *S. mansoni* also differs from bacterial collagenase as it is not affected by the presence of trace amounts of sulfhydryl compounds or other substances capable of forming metal complexes. However, the secretions of living cercariae or the ability of living cercariae to produce enzymes are inhibited by such compounds.

The activity of extracts of filariform infective larvae of *S. ratti* also shows a

9. Neuman, R. E. and Tytell, A. A. 1950, Action of proteolytic enzymes on collagen. Proc. Soc. Exp. Biol. & Med. **73:** 409–412.

10. Bidwell, E. 1950, The proteolytic enzymes of *Cl. welchii*. J. Biochem. **46:** 589–598.

close parallel to that of the proteolytic portion of *Cl. welchii* toxin. Its action on substrates is similar to that of *S. mansoni* except that it is slightly less active on cartilage and has a lower pH optimum. It differs, however, in not being sensitive to sulfhydryl-binding agents and in being even more sensitive to the presence of sulfhydryl-containing and metal-chelating agents than is the toxin. These data suggest that both the *S. ratti* enzyme and the toxin are dependent for full activity on the presence of trace amounts of divalent cations that are readily removed by such means.

Throughout this investigation it has been convenient to treat the various helminth extracts and bacterial toxin as though each contained a single enzyme and that each effect seen or measured was the result of the activity of one enzyme. It is more probable that the helminth extracts are of greater complexity like the bacterial toxin, which has been shown to have three enzymes contributing to the "collagenase" activity.[10] A wide spectrum of activities might well be expected in extracts of both *S. mansoni* and *S. ratti*, as each exhibits a rapid lysis of diverse elements of the living skin of the host during skin penetration. It has been suggested by several investigators that the cercariae of *S. mansoni* possess mucopolysaccharidase activity as does the toxin of *Cl. welchii*. It was thought possible that, on these complex substrates, mucopolysaccharidase activity might augment the proteolytic effects being measured and for this reason substances known to inhibit hyaluronidase activity were included in some experiments. These results were equivocal, as the helminth extracts were unaffected by alkalinized benzoquinone which inhibits the bacterial toxin. Ascorbic acid was without effect on the toxin and inhibited both *S. mansoni* and trypsin, but this result cannot with certainty be ascribed to mucopolysaccharidase inhibition.

The in vivo tests of substances inhibiting the helminth enzymes in vitro and presumably inhibiting penetration must be evaluated with caution. Some of the substances inhibiting the enzymes can also be cercariacidal or may possibly impair activity of the helminth larvae as do Cu^{++} or Hg^{++}. Of the substances tested in vivo without noticeably impairing larval activity only EDTA at low concentration showed marked penetration inhibition, especially in the skin penetration by larvae of *S. ratti* whose extract is also extremely sensitive to EDTA. The chelating agent also significantly inhibited skin penetration by *S. mansoni*.

SUMMARY

The cercariae of *Schistosoma mansoni* and the filariform larvae of *S. ratti* produce enzymes believed to be associated with their ability to penetrate the skin of their definitive hosts and to migrate subsequently through the tissues. Collagenase-like, proteolytic enzymes are found in each and their activities have been characterized through the use of quantitative measurements and by comparison with a bacterial collagenase and trypsin. Activity has been measured primarily as dye released from azo dye-coupled, collagen-containing, substrates and also as hydroxyproline released as amino acid or in polypeptides from collagen substrates. Various qualitative tests of activity including histochemical methods demonstrating action on native substrates in vivo have also been performed.

The activity of the helminth-derived enzymes is directly proportional to the number of organisms present in the test systems used or to the dry weight of

organisms from which the aliquot of enzyme has been extracted. This activity increases with time of incubation at 37 C. and is destroyed by heating to 60 C. The optimal pH of the enzyme of *S. mansoni* in phosphate buffer is near 7.5 while that of *Strongyloides ratti* is in the vicinity of 7.0.

The enzymes of the two helminths differ in their degree of sensitivity to various of the metal ions tested. Both helminth enzymes are inhibited by Hg^{++} and Cu^{++} and slightly activated by Ca^{++} and Mg^{++}. Agents binding sulfhydryl groups inhibit the activity of the enzyme derived from *S. mansoni* but are with little effect on that of *S. ratti*. The latter is more sensitive to chelating agents and sulfhydryl-containing compounds than is the cercarial enzyme. In contrast to trypsin, both helminth enzymes are active on cartilage and neither is affected by the presence of trypsin inhibitors. When compared to bacterial collagenase, the activity of the helminth enzymes on native collagen in vitro is slight although in vivo activity against collagen-containing tissues of the host is marked.

Various substances active in vitro in inhibiting the helminth enzymes have been tested for their ability to inhibit penetration of the skin by living larvae. One of these, a chelating agent, was capable of inhibiting penetration without otherwise causing obvious damage to the infecting organisms.

THE MAINTENANCE OF *SCHISTOSOMA MANSONI* IN THE LABORATORY

CHANG-LING LEE AND ROBERT M. LEWERT

From the Department of Microbiology, The University of Chicago, Chicago 37, Illinois

Both *Schistosoma mansoni* and *Schistosomatium douthitti* have been maintained in this laboratory since 1950. Cercariae of *S. mansoni* in large numbers were required for our studies of their enzymatic activity (Lewert and Lee, 1954 and 1956) and, of necessity, simple and effective methods of maintaining a large colony of *Australorbis glabratus* were developed. None of these methods are radical departures from reported techniques but do represent modifications in methods to permit maintenance of *S. mansoni* in the laboratory with a minimum of effort whether large or small projects are contemplated.

Aquaria.—Of the variety of containers utilized, the most satisfactory is a white porcelain-covered metal refrigerator vegetable tray, 5×9×13 inches in size. These are inexpensive, readily available, fit well in racks or on shelves, are small enough to be handled with ease and can accomodate 50 to 100 *Australorbis* (fig. 1).

The trays are filled to within 1½ inches of the top with tap water which has been passed through a column of sand and activated charcoal to remove chlorine. No sand or vegetation is included in the tray but one or more "food floats" are placed on the water surface which also provide places for the snails to deposit eggs. The floats are constructed of four corks joined by the insertion of four thin sealed glass tubes and the whole is covered with a nylon hair net having meshes large enough to allow the snails to pass through (fig. 1). These are durable and not toxic to the snails. Food is placed on top of the net floats and is supported at a level which allows easy siphoning of the bottom debris. Since the snails congregate in the area of the floats, they are readily "fished" out when needed.

All aquaria are covered with glass plates and are aerated with filtered compressed air through air "breakers." The aquarium room is illuminated 14 hours daily by fluorescent lighting fixtures and the room temperature is maintained between 25 C and 30 C. In our experience and also as reported by Stirewalt (1954), lower temperatures reduce infection of the snails and also prolong the period needed to develop cercariae. Since all of the elements mentioned are readily available, new aquaria can be established in a few minutes. Aquaria are cleaned every 2 to 4 weeks, depending on the number of snails in the tray. This is simply done by aspirating the snail feces and waste food through a water aspirator pump and refilling with dechlorinated water. It is not necessary to move the tray as debris is aspirated and water supplied through rubber tubing connected to the water supply. The tubes are equipped with plastic screens made by perforating plastic test tubes of suitable size to fit the rubber tubing. The screen allows waste to be removed but prevents loss of snails.

Preparation of snail food.—We have

Received for publication September 6, 1955.

This study has been made under the sponsorship of the Commission on Parasitic Diseases, Armed Forces Epidemiological Board and was supported in part by the Office of the Surgeon General, Department of the Army, and in part by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago.

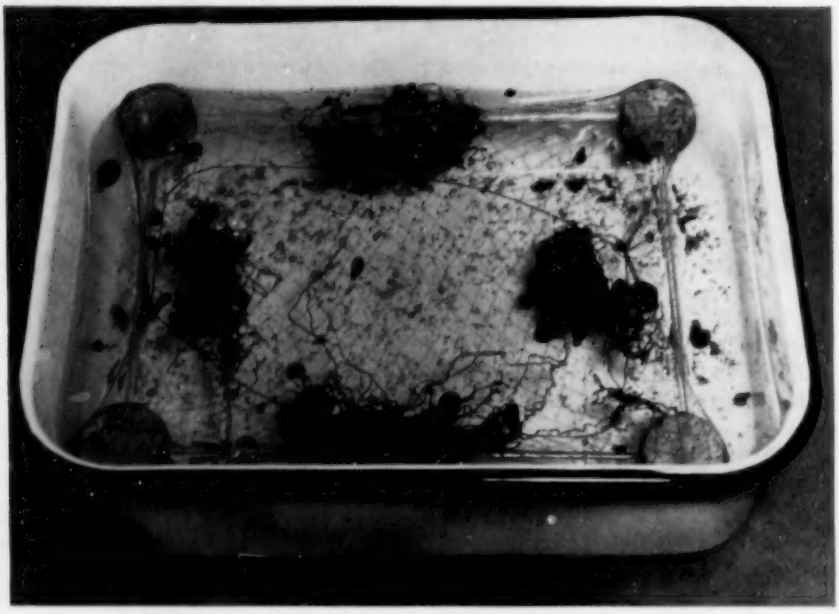


FIG. 1.—Aquarium showing food float, food, and snails. The glass cover and air bubbler usually present have been removed.

found that the calcium alginate preparation introduced by Standen (1951) and modified by Moore et al (1953) and by Sandground and Moore (1955) has many advantages when compared to other types of snail food such as lettuce or soaked maple leaves. A uniform preparation can be made in quantity and can be stored frozen until needed. It does not readily mold or deteriorate and we have used it exclusively for 2 years. However, a simpler effective method of preparing the alginate food than that previously described is as follows:

To 500 ml of water at 50 C while mixing in a Waring blendor, add slowly 10 g Cerophyl (dehydrated cereal grasses, Cerophyl Laboratory Inc., Kansas City, Mo., sieved to pass 40 mesh screen), 2.5 g powdered whole milk, 5 g powdered wheat germ (if not obtained in powdered form must be further ground dry in the Waring blendor and sieved to pass 40 mesh), and finally 5 g sodium alginate (standard grade, Algin Corporation of America, 24 State Street, New York City 4). Several batches of the well-mixed viscous liquid are transferred to a heavy-walled 2 liter filtering flask. The flask has a removable but tightly stoppered top, through which a tube leads to its bottom (fig. 2). The side arm of the flask is connected to an air line through a similar flask containing water to trap any oil present in the compressed air. The air pressure (approximately 15 lbs) is so adjusted that the alginate mixture is forced from the flask through a tapering glass tube in a continuous stream. The end of the glass tube has an opening to deliver an alginate stream about 1 mm in diameter and the stream is directed to flow into a 2% calcium chloride solution with the delivery tube one inch above the solution surface. A continuous strand

of insoluble calcium alginate food is thus formed. Some care must be taken to regulate the air pressure as too great or too little pressure results in either masses or particles of food rather than the continuous filament desired. From time to time as formed, the alginate strands are transferred from the original tray of calcium chloride solution to a second tray of 2% calcium chloride for 10 to 15 minutes and then are washed in cold running water for another 15 minutes to ½ hour. After washing, the food is transferred to 4 to 6 oz wide-mouth screw-top glass jars and these are placed in a deep freeze or freezing compartment of a refrigerator for storage. When needed one or more jars may be thawed and used. After freezing and thawing, the filaments lose some water and appear darker green than when originally prepared. By this method, 10 liters of the alginate food can be processed in 4 hours by a single individual. This amount is approximately sufficient to maintain 2000 snails for a month.

If only a small amount of the alginate food is needed, the liquid sodium alginate mixture can be dispensed into the calcium chloride solution by using a 5 ml continuous injection syringe and 18-gauge needle.

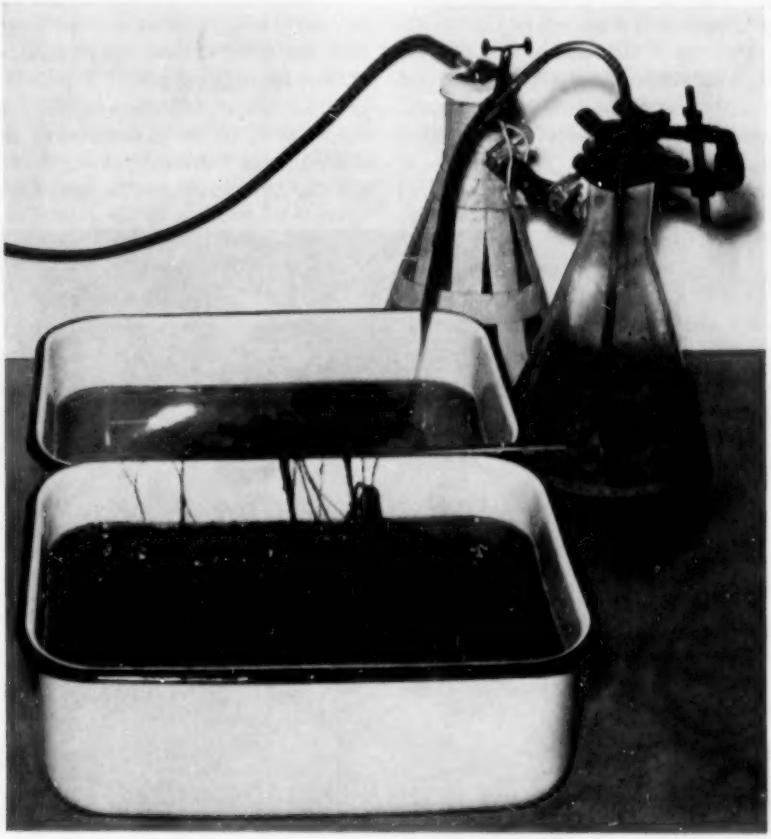


FIG. 2.—Apparatus used to prepare calcium alginate snail food in continuous filaments.

Lymnaea palustris and *Lymnaea stagnalis*, the snail hosts of *S. douthitti*, have been maintained successfully in a manner identical to that described above.

Exposure of mice to cercariae.—The method of exposing mice to cercariae is similar to that described by Luttermoser (1954), but the time required for the entire operation and the amount of handling of infectious material are decreased. An outline of the procedure is as follows:

A group of 10 to 20 infected *A. glabratus* are placed in a 250 ml beaker with 150 ml of dechlorinated water. After sufficient cercariae have been shed, they are gently decanted through a 100 mesh screen into a second beaker to obtain a cercarial suspension free of snail feces. The suspension is gently mixed and 0.2 ml aliquots are removed and counted with the aid of a binocular dessecting microscope. The volume of the suspension containing the desired number of cercariae is transferred to 10×75 mm test tubes by means of an automatic pipette such as the Alfred Bicknell pipette. The pipette has an aperture at least 2 mm in diameter to deliver the cercariae freely without injury. Dechlorinated water is added to fill the tubes within 1 cm of the top and they are placed in rows of ten in a standard 20 tube rack. This one and another one to hold 10 anaesthetized mice are placed in a 5×10×12 inch mouse cage. and the tails of the mice are placed in the test tubes (fig. 3). The mice (CF 1), 20 to 25 g in weight, are anaesthetized by an intraperitoneal injection of veterinary nembutal at a dosage of 100 mg/kg of body weight. This dosage immobilizes the mice without any physical restraint for approximately 1 hour, which is more than sufficient to allow those cercariae able to penetrate to do so (Oliver and Stirewalt, 1952 and McCarthy et al 1954). The anaesthetized

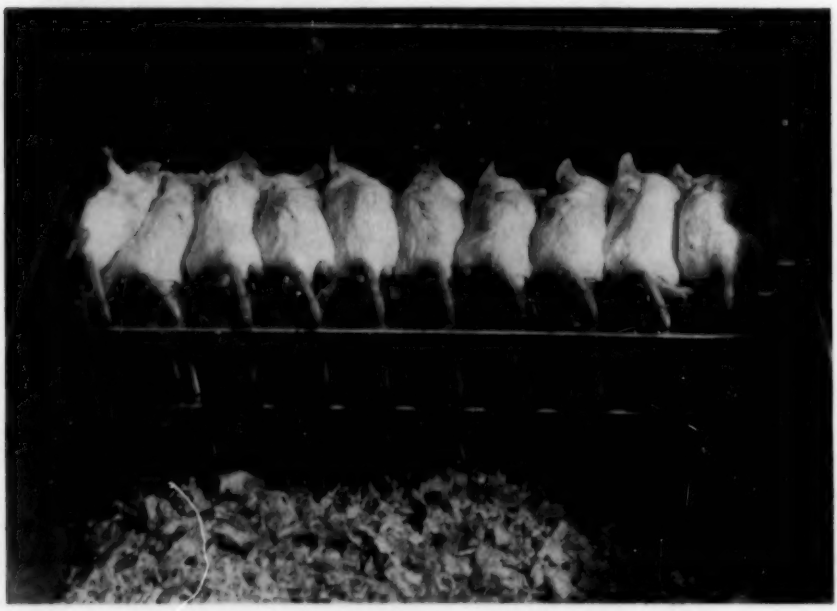


FIG. 3.—Routine method of exposure of mice to *S. mansoni* cercariae.

mice do not remove their tails from the tubes. Each cage prepared in this way is provided with food and water and closed. The animals when recovered are free to move about the cage in a normal manner. The tubes and their racks are removed the next morning or whenever convenient. Using this method with care, the anaesthetic mortality is very low and 44 to 59 schistosomes per mouse are recovered from mice exposed to 150 cercariae each.

Infection of snails with miracidia.—The methods for exposing snails do not differ materially from those reported by Kagan (1954). The yield of miracidia is greater and hatching more rapid, however, when infected tissues are ground in a Waring blendor as follows rather than in a mortar with sand. The tissue (liver or intestine) is placed in several volumes of 0.85% saline in a Waring blendor and minced for 2 minutes, centrifuged, and the sediment replaced in the blendor with several volumes of saline for another 2 minutes mincing. It is then washed three times in saline by centrifuging and placed in a side arm flask with water so that miracidia may be concentrated and recovered.

The end result of these modifications of methods used to maintain *S. mansoni* in the laboratory is a great saving of time and facilitates the maintenance of relatively large numbers of infected snails for the purpose of harvesting cercariae. No accurate count of the number of snails is recorded as it varies considerably from month to month. However, during one period of the past year, a maximum of 900 snails were shedding cercariae simultaneously. The infection record for this group is shown in table 1.

Disposal of contaminated articles.—Lysol, phenol and similar agents, although they readily kill cercariae, are not ideal as they render the cleaning of glassware and other containers difficult.

TABLE 1.—*The survival of 2 groups of snails.*

	Exposure to miracidia		
	Each snail exposed once to 5–7 miracidia	Snails exposed 3 times in groups of of 50 to 5–15 miracidia per snail	Total
Number of snails	1100	700	1800
Mortality in 50 days	18%	57%	33%
Infected and shedding cercariae at 50th day after exposure	630	270	900
Infection rate of survivors	70%	90%	75%

An ordinary laboratory glassware cleanser such as Lakeseal kills *S. mansoni* cercariae within 1 minute in a dilution of 0.1%, which is below the suggested concentration ordinarily used for cleaning (0.15 to 0.5%). All contaminated articles, therefore, are submerged in about 1% Lakeseal solution for several hours or overnight to disinfect and clean simultaneously.

SUMMARY

Methods for the routine maintenance of *Schistosoma mansoni* in this laboratory involve equipment and procedures such that large or small colonies of *Australorbis* can be grown efficiently with a minimum of effort and expenditure of time. Refrigerator vegetable trays are used as aquaria and the snails are provided only with calcium alginate food which is placed on a floating net. No sand or vegetation is necessary. The food is prepared efficiently in quantity as a continuous filament and can be stored indefinitely. The exposure of mice to cercariae as well as the problem of handling contaminated articles are discussed.

REFERENCES

Kagan, I. G., Short, R. B. and Nez, M. M. 1954, Maintenance of *Schistosomatium douthitti* (Cort. 1914) in the laboratory (Trematoda: Schistosomatium). J. Parasitol. **40**: 1–16.

Lewert, R. M. and Lee, C. 1954, Studies on the passage of helminth larvae through host tis-

sues. 1. Histochemical studies on extracellular changes caused by penetrating larvae. II. Enzymatic activity of larvae in vitro and vivo. J. Infect. Dis. **95**: 13–51.

———. 1956, Quantitative studies on the Collagenase-like enzymes of cercariae of *Schistosoma mansoni* and the larvae of *Strongyloides ratti*. J. Infect. Dis. **99**: 1–14.

Luttermoser, G. W. 1954, Studies on the chemotherapy of experimental Schistosomiasis. I. A method of detecting Schistosomaticidal activity based on response of *Schistosoma mansoni* infection in mice to fuadin therapy. J. Parasitol. **40**: 130–137.

McCarthy, D., Reinertson, J. W. and Thompson, P. E. 1954, A convenient device for exposing mice to *Schistosoma mansoni* by tail immersion. J. Parasitol. **40**: 704.

Moore, D. V., Thillet, C. J., Carney, D. M. and Meleney, H. E. 1953, Experimental infection of *Bulinus truncatus* with *Schistosoma haematobium*. J. Parasitol. **39**: 215–221.

Olivier, L. and Stirewalt, M. A. 1952, An efficient method for exposure of mice to cercariae of *Schistosoma mansoni*. J. Parasitol. **38**: 19–23.

Sandground, J. H. and Moore, D. V. 1955, Note on the rearing of *Oncomelania* spp. in the laboratory. J. Parasitol. **41**: 109–113.

Standen, O. D. 1951, Some observations upon the maintenance of *Australorbis glabratus* in the laboratory. Ann. Trop. Med. **45**: 80–83.

Stirewalt, M. A. 1954, Effect of snail maintenance temperatures on development of *Schistosoma mansoni*. J. Parasitol. **40**: 35.

ANTIBODIES IN THE RABBIT WITH DIFFERENT RATES OF METABOLIC DECAY

WILLIAM H. TALIAFERRO AND DAVID W. TALMAGE

From the Departments of Microbiology and of Medicine, University of Chicago, Chicago 37, Illinois

Besides its intrinsic interest, the rate of metabolic decay of antibody is necessary for any determination of the total amount of antibody formed during any specified time (see W. H. and L. G. Taliaferro, 1951, and Taliaferro, 1955). In general, such rates have been determined by the rate of disappearance of passively transferred homologous antibody from the serum of recipient animals. When plotted semilogarithmically, the disappearance is generally biphasic. The initial period of rapid fall, immediately following intravenous injection of the antibody, is curved in shape and is usually considered to be the result of a tissue blood equilibration. The second phase is linear and is considered to represent the true metabolic decay. The rate of metabolic decay is commonly expressed as the biological half life and can be read directly from plots of log antibody against arithmetic time or can be calculated from the following equations of declining antibody titer:

$$A_t = A_0 e^{-k_1 t}$$

$$k_1 = \frac{\ln A_0 - \ln A_t}{t}$$

and when

$$A_t = \tfrac{1}{2} A_0$$

$$t_{\frac{1}{2}} = \frac{0.693}{k_1}$$

in which A_t and A_0 are antibody titers at times t and zero, respectively, k_1 is the rate constant of antibody change, and $t_{\frac{1}{2}}$ is the biological half life of the antibody.

Dixon et al (1952) found that the biological half life of homologous γ globulin in the rabbit was 4.6 ± 0.8 days when the globulin was prepared by alcohol precipitation and 5.7 ± 1.2 days when prepared by ammonium sulfate precipitation. The smaller value was almost identical with the half lives of several rabbit antibodies which they calculated from the data of Glenny and Hopkins (1923) for diphtheria antitoxin (4 to 5 days), Heidelberger et al (1942) for type 1 antipneumococcus antibody (4.2 days) and Germuth (1951) for antiovalbumin (4.7 days). The half-life of passively transferred rabbit antibodies was found to be 5.6 days for antibovine globulin by Stevents et al (1953) and 5 to 5.4 days for antipneumococcus antibody by Humphrey and McFarlane (1954).

The present investigation grew out of our finding (Taliaferro and Talmage, 1954) that rabbit Forssman hemolysin decayed metabolically at a much more rapid rate than other rabbit antibodies as recorded in the foregoing reports or than a rabbit antibody to human γ globulin. Many of the present experiments were completed before we (Talmage et al, 1956b) discovered that both Forssman and isophile antisheep-red-cell antibodies contain at least two components: a large molecule of high

Received for publication January 23, 1956.

The work was done under contracts AT (11-1)-175 and AT (11-1)-331 between the U. S. Atomic Energy Commission and the University of Chicago. It was also supported in part by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

The authors are indebted to Dr. Lucy Graves Taliaferro for assistance throughout the work and to Mrs. Gloria G. Freter for technical assistance.

hemolytic capacity and a small molecule with little, if any, hemolytic capacity. Later work as well as a reinterpretation of our early experiments indicated that the large hemolytic molecule is associated with a short biological half life and the small molecule with a longer half life.

MATERIALS AND METHODS

Rabbits were used exclusively. They were obtained from Mr. Harold Swift's Lakeside farm in Michigan and were free of snuffles and other respiratory diseases. Most of them weighed from 2 to 2.5 kg at the beginning of an experiment. Males and females occurred approximately in equal number. They were kept at least a week in our laboratory before they were used.

A. Immunization of rabbits.—Hemolysins were prepared by injecting sheep red blood cells or heated sheep red blood cell stromata intravenously. The red cells in Alsever's fluid were gently shaken in 50 to 100 times their volume of 0.85% sodium chloride, were centrifuged for 15 minutes at 2000 r.p.m. and were standardized photometrically on the basis of hemoglobin liberated by distilled water lysis (W. H. and L. G. Taliaferro, 1950). Thus, 1 ml of a 1% suspension (approximately 1.6×10^8 red cells), after lysis by diluting 1:5 with distilled water, gives a reading of 150 on the Klett Summerson photoelectric colorimeter with a #54 filter (range 500 to 570 mμ). One ml of 1% cells as thus defined contains approximately 2.5 mg hemoglobin on the basis of dry weight as determined by Talmage and Freter (1956).

Heated sheep red cell stromata were prepared by the method of Jaroslow and Taliaferro (1956) and were autoclaved at 2 pounds pressure (approximately 103 C) for 1 hour.

The 4 following procedures were used. (1) Intact or splenectomized rabbits were injected 7 to 10 times with 1 ml of 10% red cells per kg rabbit during periods ranging from 10 to 13 days and were bled 2 to 4 days after the last injection (about 13 to 15 days after the first injection) near the time of peak titer. The rabbits were splenectomized because higher (though later) serum peak hemolysin titers were attained than in intact rabbits (W. H. and L. G. Taliaferro, 1951). (2) Intact rabbits were subjected to the same treatment as in #1 except that heated stromata were used. Both of these procedures yielded serums with high Forsman hemolytic efficiency. (Isophile antibody did not appear in measurable concentration by the 15th day in procedure #1 even though whole red cells were injected.) (3) Intact rabbits were injected 12 or more times every 2 or 3 days with 1 ml of 10% sheep red cells per kg rabbit and were bled a week after the last injection (about the 35th day after the first injection). (4) Intact rabbits were given a series of 6 to 12 injections of the same amount as in #3 (at the rate of 3 a week) followed a month later by a 2nd series consisting of 3 to 6 injections and were bled a week after the last injection. Procedures 3 and 4 yielded appreciable amounts of isophile antibody in proportion to Forssman antibody. (The latter was decreasing at the time the serum was obtained.) Isophile antibody in procedure 3 did not appear regularly until the 4th week (Talmage et al, 1956a).

Antihuman-γ-globulin serum (anti-HGG) was obtained by using Lederle's immune serum globulin #2167–98 (human γ globulin or HGG). The immunizing antigen was prepared by emulsifying in a Waring blendor 10 parts of a 0.5% saline solution of HGG with 1 part arlacel C (Ward et al, 1950) and 9 parts of bayol F (Salk and Younger, 1950) which gave a final dilution of 12.5 mg HGG/ml. Six rabbits were given from 3 to 5 injections of 50 mg HGG in 4 ml of emulsion at approximately monthly intervals. Each injection was distributed among 2 intramuscular and 2 subcutaneous sites. High titered precipitating serums were obtained by intracardiac puncture 7 days after the 3rd through the 5th injection.

B. Materials used for passive transfer.—Antiserums prepared as described under section A were injected into normal recipients. In addition, the first and second specific eluates from antiserums iodinated as described under section C were similarly injected into normal recipients. The recipients were then bled within 10 minutes and daily thereafter for a week or more. In many cases, more bleedings were made during the first 24 hours. The number of days bleedings were made depended upon the amount of antiserum injected and its titer. The serums were titrated by immunological methods (sections E and F) or by radioactivity determinations (section D).

C. Iodination of antibody for passive transfer.—Anti-HGG serums were trace-labeled with I^{131} as follows. Globulins were first precipitated from the anti-HGG serum by 50% saturation with ammonium sulfate. After dialyzing to remove the ammonium sulfate, the globulins were iodinated. Specific antibody was then separated from the other globulins by absorbing on the specific antigen and eluting with carrier anti-HGG serum at pH 7.4 by the method of Talmage et al (1954). Such preparations contained antibody of high isotopic specificity. In the preparations used in the present study, about 80% of the

radioactivity in the eluate used for passive transfer was bound to specific HGG antibody. A considerable amount of unlabeled carrier anti-HGG antibody was also present but did not affect the radioactivity counts.

The same method was used to prepare the iodinated Forssman antiserums for 5 animals in group 4 except that iodinated Forssman antibody was purified by absorbing on red cells and eluting from red cells with unlabeled, i.e., carrier, Forssman antiserum at pH 7.4 (Talmage and Freter, 1956).

For animals in groups 4 and 6, Forssman and isophile antibodies were prepared as follows. Specific iodinated antired-cell serum was separated from the other iodinated globulins by absorbing it on red cells and eluting the labeled antibody from the cell-antibody combination with carrier Forssman antiserum at pH 7.4. This 1st eluate contained I^{131}-labeled and carrier Forssman antibody as well as approximately 25% of non-antibody globulins. The carrier antibody did not affect the radioactive counts. The cell-antibody combination (remaining after the first elution) was treated a second time with carrier antired-cell serum to obtain the isophile antibody. The protein-bound label in this 2nd eluate was from 75 to 80% antibody-bound and a large proportion of the I^{131}-labeled antibody was isophile.

D. Radioactivity determinations.—The disappearance of antibody trace-labeled with I^{131} from passively immunized recipients was followed by determining γ ray emission from blood samples. Experimental rabbits were bled into weighed glass (Wassermann) tubes, and the radioactivity of the weighed blood sample (about 1 ml) in the tube was determined in a scintillation counter in connection with a single channel analyzer. Radioactivity was expressed as counts per minute per gram of whole blood.

The same counter and analyzer were used in the combining capacity determinations (section E).

E. Immunological titrations.—Hemolytic activity was titrated photometrically in 50% hemolytic units by the method described by W. H. and L. G. Taliaferro (1950) and is expressed in 50% units/ml. Briefly, this method measures the amount of serum which lyses 50% of a photometrically standardized amount of sheep red cells in the presence of four 50% units of complement.

The combining activity of antired-cell serum was titrated in 50% combining units defined as the amount of serum which gives a 50% inhibition of the uptake of an I^{131}-traced-labeled antibody (Talmage and Freter, 1956).

F. Separation of Forssman and isophile antibodies in antired-cell serums for immunological titrations.—Three methods were used: (1) Forssman antibody was removed from antiserums by absorption with heated red cell stromata, prepared as described above.

(2) Horse kidney was also used for absorption. It was prepared by removing the capsule and connective tissue, cutting into small pieces and mincing for about an hour in a Waring blendor with phosphate saline buffer. After screening out the coarse particles, the fine particulate material, suspended in about 3 liters of phosphate saline buffer, was centrifuged in a Sharples centrifuge at an average centrifugal field of 13,000×g. It was washed twice while in the Sharples, left overnight in the refrigerator and autoclaved at 2 pounds pressure (approximately 103 C) for 1 hour. It was again put in the Waring blendor for 1 hour to break up the large aggregates caused by heating, collected in the Sharples centrifuge, mixed with an excess of buffered saline and collected in a refrigerated centrifuge in an average gravitational field of 2600 × *g* for 15 minutes. The yield from one horse kidney was about 70 g wet weight.

(3) Each antiserum was divided into 4 aliquots. The first aliquot was titrated for hemolytic activity and because of the small amount of isophile antibody (2 to 9%) was considered to be Forssman hemolysin. The second one was titrated for Forssman combining capacity by inhibition of the uptake of a labeled Forssman antibody. The third aliquot was titrated for isophile combining activity by using a labeled isophile antibody and in addition adding 3.2×10^7 heated stromata per Forssman combining unit to absorb the Forssman antibody. The fourth aliquot was absorbed with heated stromata and then titrated for isophile hemolytic activity. Generally, there were two absorptions with about 5×10^8 heated stromata per hemolytic unit either at room temperature for an hour or at 37 C for half an hour.

All absorptions of Forssman antibody were continued until 2 successive ones showed no significant decrease in titer.

EXPERIMENTAL RESULTS

The fate of passively transferred rabbit Forssman and isophile antibodies against sheep red cells was followed by specific serological tests (50% hemolytic capacity and 50% combining capacity) and by specific radioactivity after trace-labeling with I^{131}. Passively transferred rabbit anti-HGG serum was followed only by the specific radioactivity

method. In figures 1 and 6, actual titers are shown. In the rest of the figures, percentages of the first (10-minute) determination are given.

As mentioned earlier, the curves of the disappearance of passively transferred antibody were predominantly biphasic when plotted semilogarithmically (cf. fig. 1 and 2). The initial relatively short decline, usually less than 2 days, was curved in shape and represented the equilibration of antibody between the blood and the tissues. The second decline of 5 to 22 days represented the actual metabolic decay and was usually linear until it neared the concentration of natural or pretransfer antibody. The longer declines were correlated with the injection of large amounts of high titered antiserums. In addition, the variation in the rate of disappearance among different rabbits of approximately the same age was small as is shown by the data in figure 1 and by the size of the standard errors in table 1. In this connection, Dixon et al (1952) reported that the metabolic half life depended upon the specific metabolic rate of the recipient.

Antisheep-red-cell antibodies

Forssman hemolytic activity, group 1.—The metabolic half life of the hemolytic activity of Forssman antibody was determined by the passive transfer of 2 types of antiserum into 27 normal rabbits. Twelve recipients were intravenously injected with 5 to 12 ml of antiserum (whole blood was used in two animals) from donors injected with heated stromata according to procedure 2, section A, in Materials and Methods. The hemolysin titers of the antiserums varied from 10,000 to 63,000 units/ml. Fifteen rabbits received antiserums from donors injected with fresh cells according to procedure 1, section A, in Materials and Methods. These donors might be expected to yield a mixture of Forssman and isophile hemolysin but actually only contained Forssman antibodies as ascertained by Talmage et al (1956a). These authors found that isophile antibody does not appear in measurable quantity until the third week in such donors and then in only a few animals and in small amounts.

The half life of hemolysin in the 27 passively transferred antiserums varied from 1.6 to 3.9 days with a mean of 2.81 ± 0.12 days (table 1, group 1). It is noteworthy that in the 15 antiserums obtained by immunization with fresh red cells it varied from 1.6 to 3.8 days (see examples in fig. 1) with a mean of 2.82 ± 0.16 days and that in the 12 anti-

TABLE 1.—*Half life in the rabbit of passively transferred (A) rabbit Forssman and isophile sheep red cell antibodies and (B) rabbit human γ globulin antibody.*

Group	Antibody	Antigen or treatment of antiserum†	Method of titration‡	Half life in days	Equilibration period		Number of rabbits
					Length in days	Percent antibody lost	
		A. Sheep red cell antiserums					
1a	Forssman hemolysin	Heated stromata	H	2.79 ± .17	0.49 ± .17	19.7 ± 5.0	12
1b	Forssman hemolysin	RBC-E	H	2.82 ± .16	0.21 ± .08	16.2 ± 1.5	15
1	(=1a and 1b)		H	2.81 ± .12	0.33 ± .08	17.7 ± 2.5	27
2	Isophile hemolysin	RBC-L (Abs F)	H	3.00 ± .33	0.62 ± .16	45.6 ± 3.4	6
3	Forssman combining	Heated stromata	C	5.56 ± .17	1.30 ± .24	46.5 ± 7.4	4
4	Forssman combining	Specific elution	I°	4.26 ± .29	1.60 ± .23	59.6 ± 2.2	8
5	Isophile combining	RBC-L (Abs F)	C	5.50 ± .25	1.80 ± .34	55.8 ± 5.4	4
6	Isophile combining	Specific elution	I°	5.60 ± .35	1.13 ± .11	61.1 ± 3.3	3
		B. Human γ globulin antiserum					
7	Anti HGG	Specific elution	I°	5.68 ± .11	1.52 ± .22	56.1 ± 1.8	6

† RBC-E, immunization with red cells and antiserum obtained early before isophile antibody appeared; RBC-L, immunization with red cells and antiserum obtained late after isophile antibody had appeared; Abs F, antiserum from which Forssman was absorbed; Specific elution, I^{131}-labeled antiserum purified by elution from antigen with carrier antibody.

‡ H, hemolytic activity in 50% units; C, antired-cell combining capacity in 50% units; I°, specific radioactivity of I^{131}-labeled antibody.

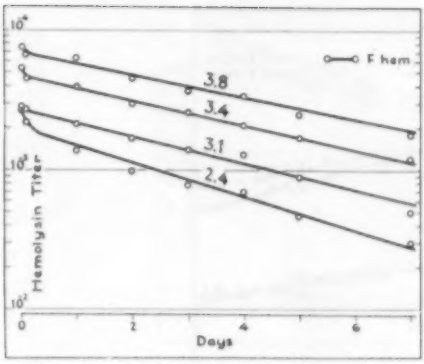


FIG. 1.—Rate of disappearance of anti-Forssman hemolytic activity (large molecules) after passive transfer to 4 rabbits. The numbers in this and all subsequent figures indicate the half life of the linear portion of the curves in days.

serums following immunization with heated stromata it was practically the same. In the latter, it varied from 1.9 to 3.9 days with a mean of 2.79 ± 0.17 days (see examples in fig. 2, solid lines).

Isophile hemolytic activity, group 2.—The rate of disappearance of isophile hemolytic activity was determined in 6 recipients.

Two recipients received intravenous injections of antired-cell serums which were prepared according to procedure 3, section A, from which the Forssman antibody was removed by the following procedure. The two antiserums originally contained, respectively, 32,000 and 84,000 hemolytic units/ml. Thirty ml of each was absorbed 3 times with 8 g (wet weight) of heated horse kidney. The serum and horse kidney mixture during the first and third absorptions were kept at room temperature for 3 hours and at about 2 C for 15 hours for the second one The mixtures were centrifuged at 5 C in an average gravitational field of 6000 $\times$ *g* after the first 2 intervals and of 12,000 $\times$ *g* after the final absorption. In each case the sediment was discarded. This treatment reduced the titer of the 1st serum to 1400 ($\approx 4.4\%$ isophile hemolysin) and of the 2nd one to 2500 ($\approx 3\%$ isophile hemolysin). Each absorbed serum was then injected into a single recipient. The disappearance of hemolytic activity in these recipients could only be followed for 3 days because the titer began to rise on the 4th day due to the active formation of antibody from a little horse kidney left in the serum.

Two other recipients received unabsorbed serums containing either 53,000 or 63,000 hemolytic units/ml serum. Their serum samples were titrated for hemolysin before and after absorption with horse kidney. A third group of 2 recipients are the ones described in the first paragraph of the next section.

The mean half life of the isophile hemolysin in these 6 rabbits was 3.0 ± 0.33 days (table 1, group 2) which is not significantly different from that of the Forssman hemolytic activity (table 1, group 1).

Forssman combining capacity, groups 3 and 4.—Two serums prepared by two courses of immunization with red blood cells were found by absorption with heated stromata to contain about 2% isophile antibody. They were given intravenously without absorption to 4 recipients. Each sample of serum from the recipients was then separated into 4 aliquots for titrations according to the 3rd method under section F. The results from one of these rabbits are shown in figure 3. The Forssman and isophile hemolytic units gave lower half life values (2.9 and 3.5 days) than the Forssman and isophile combining units (6 and 5.5 days). The mean half life of the Forssman combining capacity for the 4 rabbits in group 3 was 5.56 ± 0.17 days (table 1).

Three recipients in group 4 received eluates containing I^{131}-labeled Forssman antibody and also Forssman antiserums

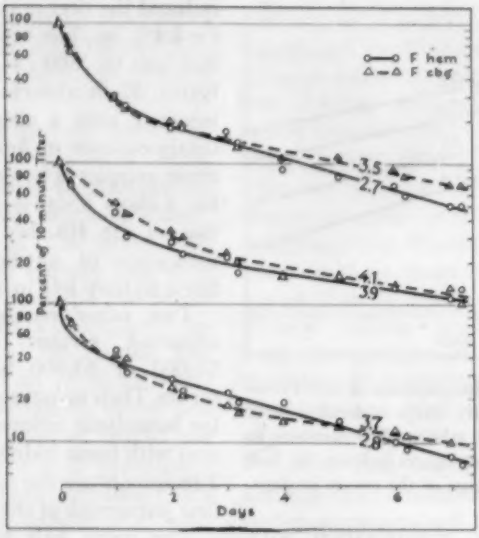


FIG. 2.—Rate of disappearance of anti-Forssman hemolytic activity and of Forssman combining capacity (a mixture of the large and small molecules) after passive transfer to 3 rabbits of 3 appropriately treated antiserums. In this figure and the next 3 figures, the 10-minute titration is considered 100%.

with a high hemolytic titer. Parallel determinations were then made on the rate of disappearance (1) of the combining capacity (hemolytic antibody of large molecular size as well as the nonhemolytic antibody of small molecular size) by radioactivity determinations and (2) of hemolytic activity by immunological determinations. The mean half life of the specific radioactivity was 3.77 ± 0.17 days. Not only was this a 35% increase over the mean determination of Forssman hemolytic activity, but the increase was consistently maintained in all 3 recipients (fig. 2). Student's method of paired comparisons indicated $P < 0.01$ for this difference.

Five additional rabbits were treated similarly except that the I^{131}-labeled antiserums contained isophile as well as Forssman antibodies (see method 3 in section A, section C, and group 6). The iodine label of the 1st eluates containing Forssman combining capacity (fig. 4A) disappeared with a mean half life of 4.43 ± 0.29 days. There was a $P < 0.05$ for the paired differences between the hemolytic activity and the specific radioactivity.

The shorter mean half life (4.26 ± 0.29 days) of the combining capacity in the 8 rabbits of group 4 which received iodinated antiserums as contrasted with the half life (5.56 ± 0.17 days) obtained by serological methods in the 4 rabbits of group 3 undoubtedly arose from the following facts. The combining capacity as determined by serological and radioactivity tests measures both large and small molecules. The antiserums used for iodination were taken from donors at about peak hemolytic titer when there was relatively much more of the large hemolytic molecules of short half life. The antiserums used in the serological tests, on the other hand, were taken later when there were fewer of the large molecules due to the fact that they

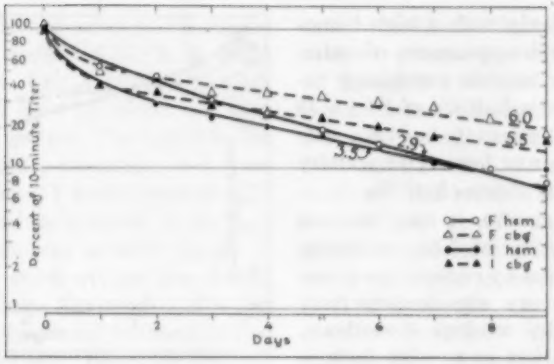


FIG. 3.—Rate of disappearance of Forssman and isophile hemolytic activities and of Forssman and isophile combining activities after passive transfer into a rabbit of an appropriate antiserum. These 4 activities are indicated consistently in the graphs as follows: Forssman hemolytic, F hem; Forssman combining, F cbg; isophile hemolytic, I hem; and isophile combining, I cbg.

are lost from the serum more rapidly and their rate of formation decreases sooner (Talmage et al, 1956a).

Isophile combining capacity, groups 5 and 6.—As shown in table 1, group 5, and as illustrated in figure 3, the mean half life of the isophile combining capacity, determined by immunological methods, for 4 rabbits was 5.5 ± 0.25 days. This figure does not differ statistically from the one obtained for the Forssman combining capacity with similar methods.

Three rabbits received the 2nd eluates of I^{131}-labeled antiserums described in the preceding section together with

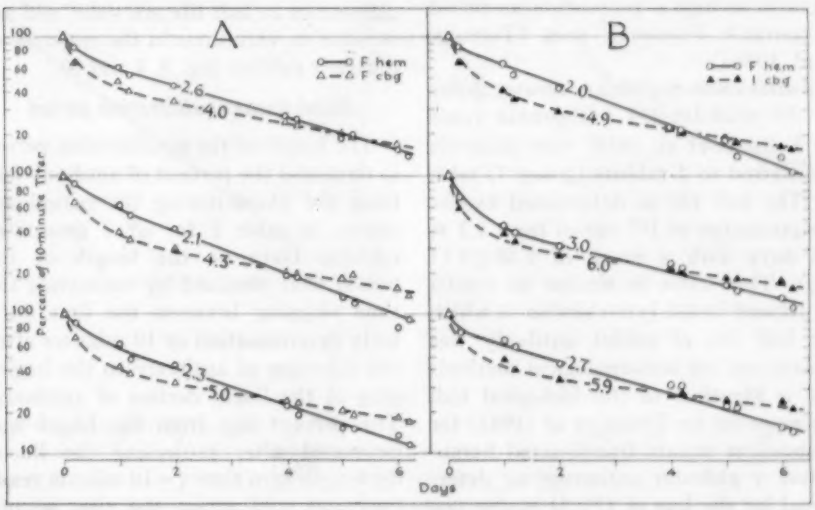


FIG. 4.—Rate of disappearance after passive transfer of 3 appropriately treated antiserums (A) of Forssman hemolytic and combining capacities in 3 rabbits and (B) of Forssman hemolytic and isophile combining activities in 3 rabbits.

Forssman antibody with a high hemolytic titer. The disappearance of radioactivity of the isophile combining capacity indicated a half life of 5.6 ± 0.35 days (table 1, group 6 and fig. 4B) while the Forssman hemolytic activity had the expected shorter half life.

From these results, it may be seen that the value for isophile combining capacity obtained by radioactive measures did not vary significantly from that obtained by serological methods. The question arises as to why there is such a good correspondence between the two methods with isophile combining and not with Forssman combining activity. We believe the difference to be due to the fact that the content in the antiserums of large short-lived molecules of the Forssman type varies markedly early and late in immunization due to the faster decay of the large molecule and an earlier inhibition of their formation. In contrast, although the large short-lived molecules of the isophile type also vary, they never occur in as high a proportion as do the comparable Forssman ones (Talmage et al, 1956a).

Anthihuman-γ-globulin serum, group 7.—I^{131}-trace-labeled antiserums (used by Talmage et al, 1954) were passively transferred to 6 rabbits (group 7, table 1). The half life as determined by the disappearance of I^{131} varied from 5.2 to 6.0 days with a mean of 5.68 ± 0.11 days. This value is similar to results mentioned in the Introduction in which the half life of rabbit antibody was determined by immunological methods and is identical to the biological half life reported by Dixon et al (1952) for ammonium sulfate fractionated homologous γ globulin antiserum as determined by the loss of I^{131}. It is also not significantly different from the values obtained for groups 3, 5 and 6 (table 1).

Two of the foregoing rabbits were injected with Forssman unlabeled anti-

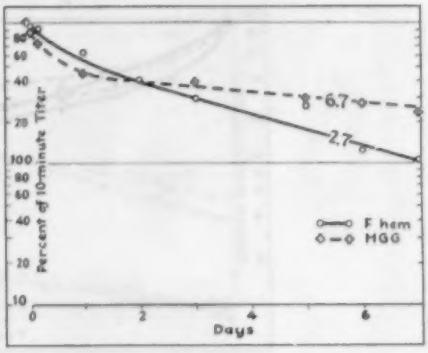


FIG. 5.—Rate of disappearance of Forssman hemolytic activity and of I^{131}-trace-labeled anti-human-gamma-globulin antibody after simultaneous passive transfer of 2 appropriate antiserums to the same rabbit.

red-cell serum along with the I^{131}-labeled anti-HGG serum. In both recipients, the Forssman hemolytic activity disappeared more rapidly than did the iodine label of the anti-HGG serum (fig. 5).

Such results with long- and short-lived antibodies demonstrate that the differences in half life are valid and are not due to variations in the response of different rabbits (fig. 3, 4 and 5).

Blood tissue equilibration period

The length of the equilibration period in days and the percent of antibody lost from the blood during the period are shown in table 1 for all 7 groups of rabbits. Data on the length of the period were obtained by estimating the time elapsing between the first antibody determination at 10 minutes after the injection of antibody to the beginning of the linear decline of antibody. The percent loss from the blood was estimated after projecting the linear decline to zero time (=10-minute reading) and subtracting the titer so obtained from the actual 10-minute reading. Although such data were only rough approximations, the Forssman hemolytic activity (associated with the

large hemolytic molecules) had a shorter equilibration period (0.33 ± 0.08 days) during which less antibody ($17.7 \pm 2.5\%$) was lost from the blood than any of the other 6 groups. The equilibration period and the percentage loss from the blood in group 1 were approximately one half the values attained in the case of isophile hemolytic activity (group 2) and about a third or less the values attained in groups 3 through 7. The difference is not significant between groups 1 and 2. On the other hand, the data for the anti-red cell combining and the anti-HGG antibodies (groups 3 through 7) were fairly consistent and were significantly higher than groups 1 or 2.

Metabolic decay of hemolysin in actively immunized rabbits

In determining the total antibody formed from the curve of serum antibody, it is important to ascertain the effect of active immunization on the rate of antibody decay. In 4 rabbits, determinations were made on the rate of disappearance of passively transferred antiserums (1) before immunization with a single injection of sheep red cells and (2) after the actively formed antibody had reached a low level. The results from one of these rabbits are shown in figure 6 and are typical for the group. (1) After an equilibration period of 0.15 days, the hemolysin passively transferred on day 0 disappeared with a half life of 3.8 days. (2) On day 28, when the passively transferred antibody had decreased to about 100 units, the animal was injected with 0.125 ml of 1% sheep red cells. Active immunity raised the hemolysin titer to 4200 units/ml serum 7 days later and the active antibody disappeared from the blood at a slower rate ($t_{\frac{1}{2}} = 7.8$ days) than after passive transfer. (3) When the titer had dropped to 710 hemolytic units/ml on day 103, the animal was given a second passive

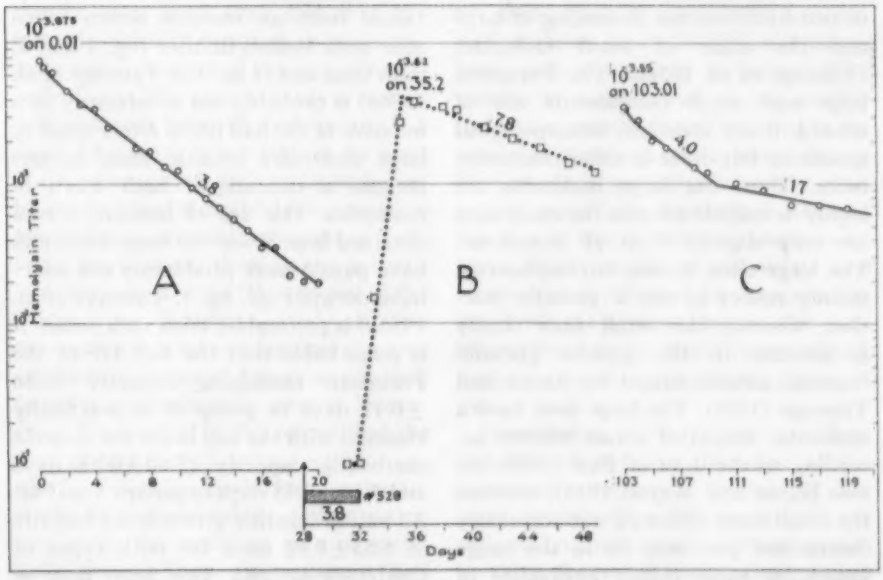


FIG. 6.—Rate of disappearance of Forssman hemolytic activity after passive transfer to a rabbit (A) before and (C) after (B) active immunization with 0.125 ml of 1% sheep red cells. Hemolysin titers are indicated at the time of transfer in A and C and at peak titer in B. The days are drawn at one fourth the scale in the other graphs.

transfer of high titered serum. The fall in serum titer was triphasic. It consisted of an equilibration period of 0.4 days, a linear decline lasting about 4 days ($t_{\frac{1}{2}} = 4.0$ days), representing largely the metabolic decay of the passively transferred serum, and a third phase of slower decline. This third phase was within the range of the active immune process just preceding the second passive transfer and undoubtedly was caused by a continuing production of antibody as a result of the injection of red cells. A daily production of about 145 hemolytic units/ml/day and a slow decline such as occurred after the 111th day would be expected with a titer of 700 50% units/ml.

DISCUSSION

The present experimental findings have to be interpreted in the light of our previous findings that the Forssman and isophile antibodies in rabbit anti-sheep-red-cell serums are each composed of two fractions: one consisting of large and the other of small molecules (Talmage et al, 1956b). The Forssman large and small components are of related, if not identical, immunological specificity but differ in other characteristics. Thus, the large molecules are highly hemolytic whereas the small ones are only slightly if at all hemolytic. The large ones, during electrophoresis, mainly collect in the T globulin fraction, whereas the small ones chiefly accumulate in the gamma globulin fraction, as ascertained by Stelos and Talmage (1956). The large ones have a molecular weight of about 900,000 according to the data of Païc (1939, see also Kabat and Mayer, 1948), whereas the small ones, although not accurately determined yet, may be in the range found for most rabbit antibodies of 157,000 to 165,000 (Kabat and Mayer, 1948). Very little work has been done on various characteristics of the large and small molecules of the isophile antibody. Stelos and Talmage (1956) have reported, however, that the large hemolytic molecules occur mainly in the γ_1 rather than the T globulin fraction during electrophoresis.

The half life of the large molecules is approximately 2.8 days for both the Forssman and isophile antibodies. This value can be accurately determined by hemolytic titrations because the small antibody molecules contribute little if any hemolytic activity to the serum.

Trace-labeling the globulin fraction, followed by absorbing the specific antibody and eluting with carrier antibody, yields eluates in which from 75 to 80% of the protein-bound I^{131} is attached to antibody (Talmage et al, 1954). The rate of disappearance of I^{131} after the passive transfer of such antibody gives an accurate measure of the half life of the antibody present. The disappearance of labeled antibody, however, obtained from an immune serum taken near peak hemolytic titer (fig. 2 and 4, dash lines and cf fig. 1 of Talmage et al, 1956a) is probably not satisfactory as a measure of the half life of either small or large molecules because these serums contain a mixture of both kinds of molecules. The use of immune serum obtained later when the large molecules have passed peak production and many have decayed (cf. fig. 1, Talmage et al, 1956a) is preferable. With such serum it is remarkable that the half life of the Forssman combining capacity (5.56 ± 0.17 days in group 3) is practically identical with the half life of the isophile combining capacity (5.50 ± 0.25 days and 5.60 ± 0.35 days in groups 5 and 6). All values together give a mean half life of 5.55 ± 0.12 days for both types of combining activity. This figure may be close to the half life value for the small molecules of both types, but will have

to be regarded only as a minimal one until purer fractions are obtained.

In a preliminary account of the present work (1954), we reported that the rate of disappearance of I^{131}-labeled antired-cell serum was longer than Forssman hemolysin and implied that the isophile hemolysin had a longer half life than the Forssman hemolysin. Further work led to the separation of the two components of each type of antibody, and we now realize that the longer half life was due to the presence of disproportionate amounts of the small relatively nonhemolytic molecule of the isophile type (Talmage et al, 1956a).

W. H. and L. G. Taliaferro (1950) found that the fall of hemolysin titer following peak titer in actively immunized rabbits increased in rate as the antigen dose was decreased. They assumed that antibody production in animals receiving a small dose (2×10^7 or 2×10^8 red cells) of antigen stopped shortly after peak titer and that the rate of antibody decrease in such animals was a measure of the rate of metabolic decay. The mean half life of hemolysin obtained from 25 rabbits was 5.5 days. This figure is too high in the light of the present work and can be explained by the fact that the rabbits continued to synthesize appreciable amounts of antibody, particularly through the activity of nonsplenic sources (cf. a review of the evidence by Taliaferro, 1955). In this connection it is interesting that the lowest limiting figure of 3 days which they found is approximately correct. In contrast, Dixon et al (1952) found that the titer of bovine-γ-globulin (=BGG) antibody, following peak titer in the actively immunized rabbit, fell at a rate similar to the expected rate of metabolic decay. Thus, the apparent half life of serum antibody for five rabbits was 5.1 ± 0.7 during 10 to 17 postimmunization days. This finding would indicate that very little antibody to BGG is synthesized after peak serum titer is reached. The difference in the rate of antibody formation to BGG following peak titer and to the Forssman antigen may reflect the difference in the persistence of a protein antigen and a carbohydrate one (cf. Heidelberger, 1953).

Our results indicating that immunization has little if any effect on the metabolic degradation of antibody are in essential agreement with those of Humphrey and McFarlane (1954) who studied antipneumococcus (type III) antibody in the hyperimmunized rabbit.

The question arises as to whether the large and small antibody molecules in each type of antiserum are metabolically related. Specifically, are the small molecules anabolically assembled into the large or, contrariwise, do the small molecules arise as degradation products from the large molecules? Neither process seems likely in view of the long period of linear decay of both the hemolytic and combining activities after passive transfer. Thus, if the smaller molecules, provided they occurred in appreciable numbers, were being assembled into large ones, the hemolytic component would be augmented less and less due to the decrease in small molecules. This situation would result in a sigmoid decay curve of hemolytic activity. With the reverse process, the combining decay curve would be sigmoid. A definitive answer to these questions awaits further work.

Our studies on the initial equilibration period indicate that less of the Forssman component containing the large hemolytic molecules of anti-red cell antibodies leaves the blood stream and that the period itself is shorter than for either type of the smaller molecules or for anti-HGG antibody. That the isophile component containing the large

hemolytic molecules may hold an intermediate position is questionable and needs more work.

CONCLUSIONS

1. Rabbit antibodies to sheep red cells or to heated sheep red cell stromata were studied following passive transfer of appropriate antiserums to nonimmunized rabbits. There were 4 antibodies involved, i.e., those of Forssman and isophile specificity, each of which consists of 2 components containing large hemolytic and small, relatively nonhemolytic molecules. The disappearance of passively transferred antibody was followed by immunological titrations of 50% hemolytic units and of 50% combining units or by radioactivity determinations of antibody which before transfer had been trace-labeled with I^{131} and purified by specific elution from red cells.

a. The biological half life of the hemolytic activity associated with the large antibody molecules of both Forssman and isophile specificity was the same, i.e., 2.81 ± 0.12 days for Forssman and 3.0 ± 0.33 days for isophile.

b. The half life of the small antibody molecules of both the isophile and Forssman type was about 5.55 ± 0.12 days. This figure, however, may be too small as the antiserums contained some large molecules.

c. Determinations of half lives by specific radioactivity gave essentially the same results as determinations by serological methods.

d. Active immunization had little, if any, effect on the metabolic degradation of passively transferred hemolysin.

2. The biological half life of rabbit antihuman-γ-globulin serum when passively transferred to rabbits as followed by I^{131}-label was 5.68 ± 0.11 days.

3. Antibodies with a slow rate of metabolic decay and a small molecular weight showed mean initial equilibration periods of 1.1 to 1.8 days during which up to 60% of the antibody left the blood. In marked contrast, those with a high rate of metabolic decay and a large molecular weight showed shorter equilibration periods during which less antibody disappeared from the blood.

REFERENCES

Dixon, F. L., Talmage, D. W., Mauer, P. H. and Deichmiller, M. 1952, The half-life of homologous gamma globulin (antibody) in several species. J. Exp. Med. **96**: 313–318.

Germuth, F. G., Oyama, J. and Ottinger, B. 1951, The mechanism of action of 17-hydroxy-11-dehydrocorticosterone (compound E) and of the adrenocorticotropic hormone in experimental hypersensitivity in rabbits. J. Exp. Med. **94**: 139–170.

Glenny, A. T. and Hopkins, B. E. 1923, Duration of passive immunity. J. Hyg. **22**: 37–51.

Heidelberger, M. 1953, Persistence of antibodies in man after immunization. In: The Nature and Significance of the Antibody Response. Ed. by A. M. Pappenheimer, Jr., Columbia University Press, New York, pp. 90–101.

Heidelberger, M., Treffers, H. P., Schoenheimer, R., Ratner, S. and Rittenberg, D. 1942, Behavior of antibody protein toward dietary nitrogen in active and passive immunity. J. Biol. Chem. **144**: 555–562.

Humphrey, J. H. and McFarlane, A. S. 1954, Rate of elimination of homologous globulins (including antibody) from the circulation. Biochem. J. **57**: 186–191.

Jaroslow, B. N. and Taliaferro, W. H. 1956, The restoration of hemolysin-forming capacity in X-irradiated rabbits by tissue and yeast preparations. J. Infect. Dis. **98**: 75–81.

Kabat, E. A. and Mayer, M. 1948, Experimental immunochemistry. Charles C Thomas, Springfield, Illinois. 547 pages.

Palc, M. 1939, Ultracentrifugation of hemolysin. Determination of its sedimentation constant and molecular weight. Bull. soc. chim. biol. **21**: 412–421.

Salk, J. E. and Younger, J. S. 1950, Antibody in yolk of eggs and in serum from hens inoculated with viruses of influenza and poliomyelitis. Federation Proc. **9**: 390.

Stelos, P. and Talmage, D. W. 1956, Electrophoretic and ultracentrifugal studies of rabbit antiserum to sheep red blood cells and to boiled sheep RBC stromata. Federation Proc. **15**: 615.

Stevens, K. M., Gray, I. and Schwartz, M. S. 1953, Catabolism of antibody and of serum albumin and globulin. Am. J. Physiol. **175:** 147–152.

Taliaferro, W. H. 1955, Studies in antibody formation. In: Some Physiological Aspects and Consequences of Parasitism. Ed. by W. H. Cole, New Brunswick, N. J., Rutgers Univ. Press, pp. 50–75.

Taliaferro, W. H. and Taliaferro, L. G. 1950, The dynamics of hemolysin formation in intact and splenectomized rabbits. J. Infect. Dis. **87:** 37–62.

———. 1951, The role of the spleen in hemolysin production in rabbits receiving multiple antigen injections. J. Infect. Dis. **89:** 143–168.

Taliaferro, W. H. and Talmage, D. W. 1954, Two antibodies in rabbits with different rates of metabolic decay. Federation Proc. **13:** 514.

Talmage, D. W., Baker, H. R. and Akeson, W. 1954, The separation and analysis of labelled antibodies. J. Infect. Dis. **94:** 199–212.

Talmage, D. W. and Freter, G. G. 1956, The blocking of labeled antibody uptake as a measure of combining capacity of anti-red cell serums. J. Infect. Dis. **98:** 277–292.

Talmage, D. W., Freter, G. G. and Taliaferro, W. H. 1956a, The effect of repeated injections of sheep red cells on the hemolytic and combining capacities of rabbit antiserums. J. Infect. Dis. **98:** 293–299.

———. 1956b, Two antibodies of related specificity but different hemolytic efficiency separated on the basis of sedimentation in a centrifugal field. J. Infect. Dis. **98:** 300–305.

Ward, R., Rader, D., Lipton, M. M. and Freund, J. 1950, Formation of neutralizing antibody in monkeys injected with poliomyelitis virus and adjuvants. Proc. Soc. Exper. Biol. & Med. **74:** 536–539.

EXPERIMENTAL INFECTIONS OF NATIVE ANIMALS WITH *PASTEURELLA TULARENSIS*

GORDON STAGG, WILLIAM S. TANNER, AND JOHN LAVENDER

During the period July 18 to September 9, 1952, sporadic epizootics in parts of the Great Salt Lake Desert were associated with the presence of tularemia in native animals. *Pasteurella tularensis* was cultured from ectoparasites (ticks, fleas, lice) of the black-tailed jackrabbit, *Lepus californicus*, the antelope ground squirrel, *Citellus leucurus*, and the white-footed deer mouse, *Peromyscus maniculatus* (Woodbury and Parker, 1953). The relationship between tularemia and the epizootics was not clearly revealed nor has it been fully clarified in the literature (Foshay, 1950). How the pathogen is maintained in the native fauna, how it is transmitted from animal to animal, and how it reappears from time to time are questions at best only partly understood.

Records of animals naturally infected with tularemia are given by Burroughs et al (1945). Since the relative susceptibility of the various animals listed is not known, the present study of experimental infection was undertaken, using native animals of the Utah West Desert area.

METHODS

Experimental animals were either reared or held after trapping. Infections were induced by three methods: (1) subcutaneous inoculations (2) contaminated food, and (3) aerosol exposures. Inoculation doses of 50 to 1000 virulent organisms (guinea pig LD_{50} value, 1 organism) were employed. Contaminated food was provided by wetting cereals and carrots with a suspension containing approximately 10^8 organisms/ml, using approximately 1 ml/carrot and 1 ml/per square inch patty of cereal.

Aerosol exposures for small rodents were provided in an exposure tube similar to the Henderson tube (Henderson, 1952) in which the dosage, calculated by breathing rate, approximated 500 organisms per animal. The cloud was generated from a Wells all-glass atomizer (Navy type) under a pressure of 10 to 12 pounds per square inch. A negative pressure was maintained in the exposure tube by using two all-glass impingers at the opposite end of the tube, each having a critical orifice which allowed 12.5 liters of air per minute to pass through a gel saline solution. A vacuum equal to 15 inches of mercury was maintained at the impingers.

The larger animals (coyotes and rabbits) were exposed in an improvised chamber fashioned from a 15-gallon drum which was substituted for the smaller exposure tube in apparatus already described.

After exposure, the animals were observed for varying periods up to 43 days for the development of symptoms or death. Blood taken from coyotes before and after exposure was treated and used in serological studies; agglutination titers were recorded as the reciprocal of the highest dilution showing 2-plus agglutination. Following death, animals were autopsied and observed for gross pathology. Spleen, liver and heart cultures were made on blood cysteine glucose agar. Liver, spleen, lymph node and heart and lung tissue samples were taken for histopathological studies.

EXPERIMENTS

The experimental animals were: black-tailed jackrabbit, *Lepus californicus deserticola;* antelope ground squirrel, *Citellus leucurus leucurus;* long-tailed pocket mouse, *Perognathus formosus incolatus;* Ord kangaroo rat, *Dipodomys ordii pallidus;* chisel-toothed kangaroo rat, *D. microps bonnevillei;* deer mouse, *Peromyscus maniculatus sonoriensis;* desert wood rat, *Neotoma lepida lepida;* and the coyote, *Canis latrans lestes.*

Infection by inoculation

Fourteen native animals of three species and four guinea pig controls were infected with a virulent strain of *Past. tularensis* (Schu). The results show that

Received for publication January 23, 1956.

This work was supported by U. S. Army Chemical Corps Contract DA-18-064-CML-2639 with the University of Utah.

TABLE 1.—*Tularemia mortality by subcutaneous injections with 50 organisms of* Past. tularensis.

Species	Infected total per number exposed	Deaths		
		Day of death		
		4th	5th	6th
Ord kangaroo rat	3/3		3	
Chisel-toothed kangaroo rat	4/4	2	1	1
White-footed deer mouse	7/7		7	
Guinea pig (controls)	4/4	4		

all died between the 4th and 6th day following inoculation (table 1). Tissues from the dead animals were culture-positive.

An attempt was made to follow the progress of infection in 50 deer mice infected with a 20-fold higher dose (1000 cells), but death of all the animals by the 4th day limited the study to this period. At autopsy, the animals were observed for gross pathology. Positive isolation of *Past. tularensis* was obtained from each of the dead animals. Table 2 illustrates the progress of infection observed using four infected and one uninfected control animals per day during a 4-day period. This table is interpreted to mean that the disease acts rapidly enough to prove fatal before serological resistance can be developed.

Infection by contaminated food

Eighteen native animals of four species and four guinea pig controls were given contaminated foods as described. Only two of the chisel-toothed kangaroo rats contracted the disease and died (table 3). There is no evidence that the others became infected. Control animals inoculated subcutaneously with 50 organisms from the same source died of infection.

TABLE 3.—Past. tularensis *infections by contaminated food.*

	Infected	Deaths		Inoculated controls
	Total per number fed	Day of death		Total per number killed
		4th	5th	
Ord kangaroo rat	0/3			3/3
Chisel-toothed kangaroo rat	2/4	1	1	4/4
White-footed deer mouse	0/7			7/7
Desert wood rat	0/4			
Guinea pig controls	0/4			4/4

Infection by aerosol exposures

Twenty-eight animals of four species of native rodents and six guinea pig controls were exposed to aerosols in the small tube exposure chamber, and three male coyote pups, one black-tailed jackrabbit and six guinea pig controls were exposed in the modified drum chamber (tables 4 and 5).

DISCUSSION

All infected rodents and rabbits succumbed in 3 to 6 days. No difference in

TABLE 2.—*Progress of tularemia infection in white-footed deer mice.*

Date Sept.	Days after exposure	No.	Pathology	Culture		
				Heart	Liver	Spleen
8	1	1	Spleen slightly enlgd., liver enlgd.	–	–	–
8	1	2	Spleen and liver normal	–	–	–
8	1	3	Spleen and liver normal	–	–	–
8	1	4	Spleen enlarged; liver dark	–	–	–
8	1	5-C	Spleen and liver normal	–	–	–
9	2	6	Spleen mottled; liver normal	–	–	+
9	2	7	Spleen enlgd. & mottled; liver normal	+	+	+
9	2	8	Spleen enlgd. & mottled; liver dark	–	–	–
9	2	9	Spleen enlgd. & mottled; liver normal	–	–	–
9	2	10-C	Spleen and liver normal	–	–	–
10	3	11	Spleen very large; liver normal	+	+	+
10	3	12	Spleen enlarged; liver normal	+	+	+
10	3	13	Spleen enlarged; liver normal	+	+	+
10	3	14	Spleen enlarged & positive; other tissue and liver soft	+	+	+
10	3	15-C	Spleen and liver normal	–	–	–

Daily developments of the disease in 50 *Peromyscus maniculatus* after subcutaneous inoculation at 10 A.M., Sept. 7, 1954, with 1000 organisms each. Four infected animals and one control were sacrificed each day. All infected animals were dead before 9 A.M., Sept. 11. Agglutinin titers were all negative. C = control.

TABLE 4.—Past. tularensis *infections by aerosols.*

Species	Dose inhaled	Deaths		Infected
		Day of death		Total per number exposed
		4th	5th	
Black-tailed jackrabbit	*	1		1/1
Antelope ground squirrel	500	2	3	5/5
Ord kangaroo rat	500	1	4	5/5
Long-tailed pocket mouse	500	4	2	6/6
White-footed deer mouse	500	12		12/12
Guinea pig controls	500	3	3	6/6
Coyote pups	*	0		3/3†
Guinea pig controls	500	6		6/6

* Dose not calculated; cloud concentration similar to that used on rodents.
† Appearance of agglutinating antibodies taken as presumptive evidence of infection.

the susceptibility of the native animals and the control animals was apparent.

The rapid progress of infection in deer mice, terminating by the 3rd day, was indicative of a dosage so overwhelming as to terminate fatally before any agglutination reaction was measurable.

The failure to infect 20 of 22 native rodents and guinea pigs given contaminated food raises several questions relating both to the survival and virulence of the agent and to the host mechanisms of resistance. These questions include: (1) Was the dosage sufficient to constitute a minimal infection dose? (2) Did the contaminating organisms survive to enter the digestive tract? (3) Was the virulence impaired by drying on the food (100% infection by the same organism via inoculation and aerosol exposure)? (4) Did the organism survive in the feces? (5) What role, if any, might endoparasites play in opening the intestinal wall to infection?

The results of the coyote exposures are in decided contrast with the experiments of Parker (1926) in which 3 coyote pups fed on Belgian rabbits and guinea pigs dead of tularemia, died at 53, 22 and 13 days respectively, after the first feedings. In these, low agglutination reactions (1:40 to 1:320) were observed between 2 and 3 weeks after exposure.

As shown in table 4, the three coyote pups survived aerosol exposure. That all three were infected by aerosol shown to be lethal for rodents is indicative that the coyote is less susceptible to tularemia than rodents. Coyote no. 3 showed no recognizable symptoms of disease at any time, but no. 1 and no. 2 did, and all three showed serological reactions (table 5).

TABLE 5.—Past. tularensis *agglutination reactions in coyotes exposed to aerosols.*

Days after exposure	Coyote		
	1	2	3
0	0	1:20	0
10	1:640	1:80	1:640
14	1:640	1:80	1:640
18	1:640		1:640
20		1:320	
24	1:640		
42		1:80	
43	1:640		

There was no cross agglutination with *Brucella suis* antigen.

Coyote no. 1 appeared in good health when the study began. Three days after exposure, he became listless, his reflexes were slower, his breathing shallow and irregular, and his fur became raised and rough. From the 3rd to 19th day he did not eat well; he became more inactive and emaciated. From the 4th to 8th days, there was a fever present. On the 20th day the food was changed from prepared dog foods to freshly killed healthy guinea pigs, and the coyote gradually recovered his health.

Coyote no. 2 was listless and had a poor appetite even before infection. Its response to infection was much less pronounced than no. 1, but more so than no. 3, which gave no outward appearance of being infected. The serological responses given in tables 4 and 5 indicate that all three responded to the infection with agglutination reactions.

SUMMARY

All rabbit and rodent specimens actually infected with *Pasteurella tularensis* died within 3 to 5 days. This mortality indicates that the organism used in the experiments was sufficiently virulent to cause death before the physiological defenses of the host could be mobilized. Failure to infect 20 out of 22 rodents via contaminated food is not explained by the experiments.

Two of three coyote pups exposed to aerosols of *Past. tularensis* showed obvious symptoms of infection, and all three showed serological evidence (agglutinin titer) of infection. Obviously they are less susceptible to tularemia than rodents.

REFERENCES

Burroughs, A. L., Holdenried, R., Longanecker, D. S. and Meyer, K. F. 1945, A list of vertebrates known to be naturally infected with tularemia, including the earliest published infection record from each species. J. Infect Dis. **76**: 117–118.

Foshay, L. 1950, Tularemia. Ann. Rev. Microbiol. 313–330.

Henderson, D. W. 1952, An apparatus for the study of airborne infection. J. Hyg. **50**: 53–68.

Kunkel, G. M. 1930, Report of a case of tularemia contracted from a coyote, *Canis lestes*, in New Mexico. Pub. Health Rep. **45**: 439–440.

Parker, R. R. 1926, Susceptibility of the coyote (*Canis lestes*) to tularemia. Pub. Health Rep. **41**(28): 1407–1410.

Woodbury, A. M. and Parker, D. D. 1953, Studies of tularemia, *Pasteurella tularensis*. Univ. of Utah. Ecol. Res. Special Rep. no. 2.

EFFECTS OF HYPOXIA AND INTERCURRENT INFECTIONS ON INFECTIONS BY *PLASMODIUM BERGHEI* IN RATS

FRANCIS W. HUGHES* AND ARTHUR L. TATUM

From the Department of Pharmacology and Toxicology of the University of Wisconsin, Madison, Wisconsin

Studies of infections by *Trypanosoma* in rats and *Plasmodium cathemerium* in canaries under the conditions of stress caused by an environmental reduction in tension of oxygen have been reported.[1–3] They have demonstrated that the host's defensive mechanisms are significantly diminished by hypoxia corresponding to that of altitudes of 19,000 feet or greater. The investigation reported herein was undertaken in order to determine whether or not there occurs a significant alteration of parasitemia in the albino rat infected by *Plasmodium berghei* under the adverse conditions of (a) an atmosphere deficient in oxygen such as in a low-pressure chamber and (b) an added intercurrent infection by another protozoan, namely *T. lewisi*.

MATERIALS AND METHODS

Adult male or female Sprague-Dawley rats weighing from 90 to 110 g were used in the following studies. The strain of *P. berghei* was obtained from Dr. G. R. Coatney of the National Institutes of Health, and was maintained in white rats or mice and transferred at 7- to 10-day intervals by intraperitoneal inoculations. The strain of *T. lewisi* was kindly supplied by Dr. Lysenko of our department of Medical Microbiology. The decompression chamber with its modifications has been previously described.[4]

Blood for stained films was obtained from the tail vein of the rat. The films were air-dried, fixed in methyl alcohol, and stained with Kingsley stains.[5] At least 500 erythrocytes were counted and the number of cells containing one or more of the plasmodia were noted and reported as percentage of parasitization of erythrocytes. Pluriparasitism was of equal degree in the hypoxic and control groups so we have considered an erythrocyte to be infected if one or more parasites had entered the stroma of the cell.

EXPERIMENTAL

In figure 1 may be seen the courses of infection which were charted on two rats (no. 10 and 11) which were placed in the decompression chamber immediately after an intraperitoneal inoculation of a saline suspension calculated to contain 4.5×10^6 organisms of *P. berghei*. The pressure within the chamber was slowly reduced through a period of 18 to 24 hours to a simulated altitude of 19,000 to 20,000 feet and maintained at this pressure for the duration of the experiment. The courses of infection were also charted for two rats (no. 12 and 13) that received the same amount of this protozoal inoculum but were maintained under normal atmospheric conditions (alt. 860 ft.) of the room throughout the period of infection.

The resultant infections of the hypox-

Received for publication February 13, 1956.

* Present address: Department of Biochemistry and Pharmacology, Indiana University School of Medicine, Indianapolis, Indiana.

1. Hanson, R. O. and Tatum, A. L. 1952, Drug, host and parasite interrelationships in the treatment of avian malaria (*Plasmodium cathemerium* in canaries). J. Infect. Dis. **90**: 105–109.
2. Hughes, F. W. and Tatum, A. L. 1955, The effects of hypoxia on infections with *Plasmodium cathemerium*. J. Infect. Dis. **97**: 231–237.
3. Hughes, F. W. and Tatum, A. L. 1956, Effects of hypoxia of rats infected by *Trypanosoma lewisi*. J. Infect. Dis. **98**: 127–132.
4. Loehning, R. W., Zapata-Ortiz, V., Hughes, F. W. and Tatum, A. L. 1953, Effects of cocaine on rats subjected to low tensions of atmospheric oxygen. J. Pharmacol. & Exper. Therap. **108**: 80–86.
5. Kingsley, D. M. 1935, A new hematological stain. I. Constituents and methods of use. Stain Technol. **10**: 127–133.

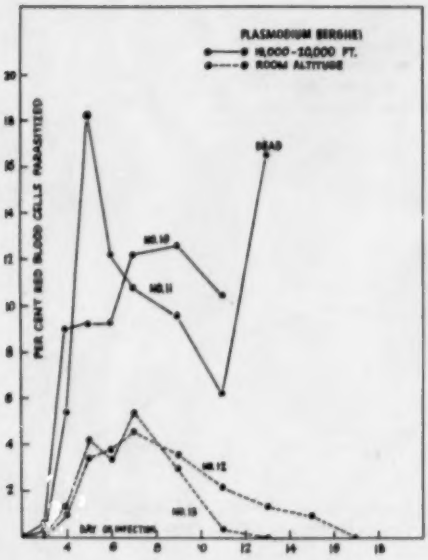


FIG. 1.—Degree of parasitemia in rats infected with *P. berghei* in room atmosphere and under conditions of simulated high altitude. Animals were exposed to hypoxia on 1st day of infection and maintained at this "altitude" thereafter.

ic rats were more sudden in onset, rose to a higher degree and at a more rapid rate than those of the control rats in room atmosphere, and, furthermore, this resultant high degree of parasitemia was maintained for a longer period and proved to be lethal for both hypoxic rats.

The immune responses appear to be adequately established after the critical peak of parasitemia occurs. After this point, if the infection in rats begins to decrease there is usually a post-climactic progressive decline leading to apparent negativity. Twelve rats were therefore selected that had been inoculated 10 days previously, each with the same calculated number of organisms. When the degree of parasitemia was in a state of progressive decrease, nine of these rats were placed in the "altitude" chamber wherein the pressure was slowly reduced through a period of 18 to 24 hours to a simulated altitude of 19,000 to 20,000 feet and maintained at this level for the duration of the experiment. The other three rats serving as controls were left in the normal air of the room for a similar period of time. The mean values of the percentage of parasitemia of each of these two groups are illustrated in figure 2. This graph demonstrates that the subjection of the near-recovered rats to a deficiency of oxygen effected a sudden, renewed rise in parasitemia and then, as rapidly, a fall in the grade of infection. Two rats died after exposure to this simulated altitude for 4 days and one expired after 6 days. The graphed parasitic density illustrates that the renewed infection was short-lived and that the parasites were visible in the blood only one day longer than in those rats that were kept under normal atmospheric conditions.

Since the crisis has been defined as the transition from natural to acquired

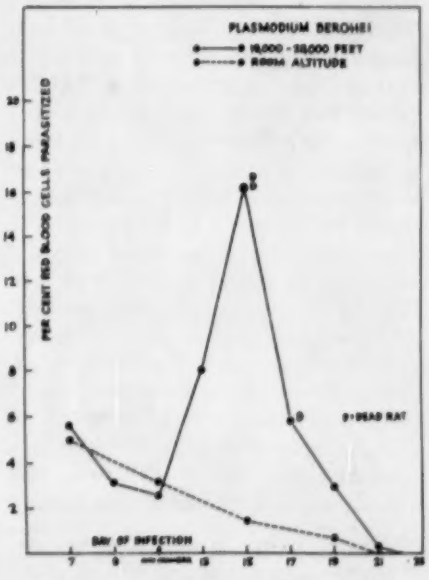


FIG. 2.—Exposure of rats to hypoxia when animals were in post-climactic recovery stages of infections by *P. berghei*.

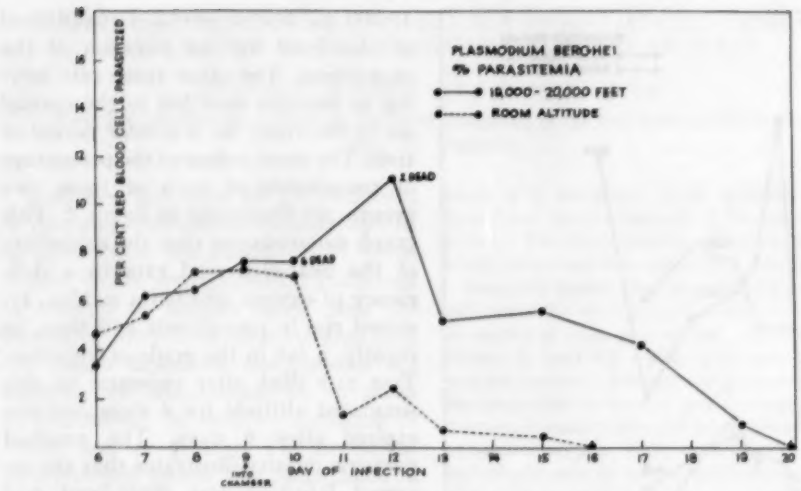


FIG. 3.—Mean degree of parasitemia by *P. berghei* in rats exposed to simulated altitudes at peak of infection as compared with rats maintained at room atmospheric conditions (860 ft.).

immunity in avian malaria,[6] a study was undertaken of the effect of inhibition or suppression of the defenses of the body by the stress of hypoxia, at the time of the crisis in infections by *P. berghei*. Twelve infected rats were put into the chamber and the pressure reduced to that of a simulated altitude of 19,000 to 20,000 feet at the time at which they were at their highest level of parasitemia. Six control rats were kept in the normal atmospheric conditions of the laboratory for the extent of the infection. In figure 3 are charted the mean values of infections of these two groups of rats. Each of the rats had originally received an equal amount of a saline-blood suspension of plasmodia. The simulated altitude caused the death of 6 of the 12 rats after 24 hours at this "altitude," although there was no demonstrable alteration in parasitemia by this time. The hypoxic atmosphere within the chamber obviously produces effects which are additive to those of the anemia caused by the malaria, thus increasing the liabilities of each. Two other hypoxic rats died after 2 days in the chamber, at which time the mean value of parasitemia had risen sharply to over 13 percent of the erythrocytes parasitized. The mean values of parasitemia of the control rats in the room were charted similarly, and it can be seen that there was the anticipated fall in parasitemia and a gradual transition to a state of negativity of the blood film by the 16th day. The parasitemia of the surviving hypoxic rats also decreased but the return to "negativity" did not occur until the 20th day of infection.

If an intercurrent protozoal infection were subject to a similar defensive mechanism as that against *P. berghei*, one would anticipate an increase in severity of infection of either one or the other or both of the protozoal infections. The response of the host to infections by *T. lewisi* has been studied very extensively by others. This flagellate promotes a response in the host which has

6. Taliaferro, W. H. and Mulligan, H. W. 1937, The histopathology of malaria with special reference to the function and origin of the macrophages in defense. Indian Med. Res. Mem. 29: 1–138.

demonstrable humoral characteristics. Two antibodies have been demonstrated, one antireproductive and the other trypanocidal. The intercurrent infections of *T. lewisi* and *P. berghei* were studied in order to determine whether or not the resultant infection by the one protozoan might be altered by that of the other.

Three rats were inoculated intraperitoneally with a suspension of *P. berghei* calculated to contain 5.4×10^6 organisms. Another group of six rats also received the same size of inoculum of *P. berghei* but in addition each animal received, also intraperitoneally, 1 cc of a suspension of *T. lewisi* containing an average of one trypanosome per high power microscopic field. Figure 4 illustrates the mean courses of infection of *P. berghei* under each of these conditions. The rats infected with *P. berghei* alone reached a peak infection on the 8th day and this was followed by a gradual return to a "negative state" by the 15th day of infection. The six rats infected by both *P. berghei* and by *T. lewisi* had a definitely augmented grade of malarial parasitemia, which continued to rise beyond the time of the critical level of the controls and reached an extremely high level by the 15th day, at which time the singly infected controls were negative. After this time there was a rapid drop in parasitemia, but two additional rats expired in spite of this apparent drop in the degree of infection.

Figure 5 illustrates the mean degree of infection with only *T. lewisi* as compared with the doubly infected rats. There is no significant difference in the mean parasitemic level of these two groups until the 11th day of infection at which time the rats, infected with *T. lewisi* only, had a parasitemia which declined to a low level and continued to do so, while in the rats that had also been inoculated with *P. berghei* the trypanosomemia had continued to increase. The previous figure (fig. 4) illustrates that at the same time the severity of infection with *P. berghei*, in the doubly infected rats was increasing, the rats infected with berghei alone continued the downward phase towards recovery. As previously mentioned, one

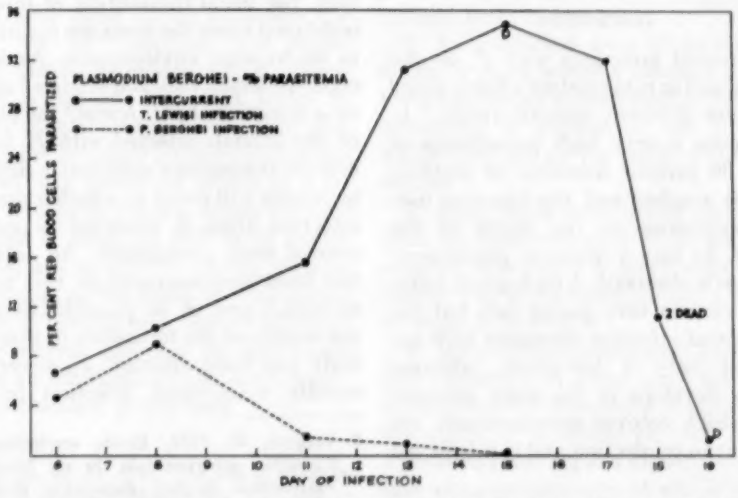


FIG. 4.—Mean degree of parasitemia by *P. berghei* in dually infected rats (*P. berghei* plus *T. lewisi*) and mean degree of parasitemia in singly infected rats (*P. berghei*).

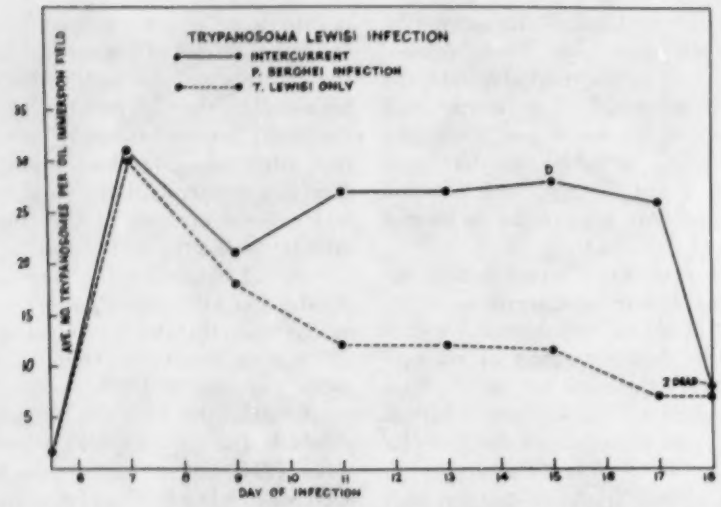


FIG. 5.—Mean degree of infection by *T. lewisi* in dually infected rats (*T. lewisi* and *P. berghei*) and mean degree of infection in singly infected rats (*T. lewisi*).

of the rats with the combined infections died on the 15th day and two on the 18th day of infection. Both groups of rats had approximately the same degree of parasitemia (*T. lewisi*) by the 18th day of infection and thereafter, with no further fatalities.

DISCUSSION

Untreated infections with *P. berghei* in mice and in rats produce effects which are quite different quantitatively.[7] In most mice a very high parasitemia of 50 to 90 percent infection of erythrocytes is reached and the infection usually terminates in the death of the animal. In rats a different parasitemic response is observed. A high-grade infection occurs in very young rats but the intensity of infection decreases with age so that only a low-grade infection usually develops in the older animals. Rats which recover spontaneously are resistant to reinfection and it is believed that the animals are truly immune and do not display a chronic infection since a transfer of blood to recipient mice or rats does not produce an infection.

In infections of rats with *P. berghei* it has been demonstrated, as reported herein, that in the course of the infection, the usual diminution of infection is delayed when the hosts are maintained in an hypoxic environment. As in the reported avian malarial studies,[2] as well as in infections by *T. lewisi*,[3] the placing of the animals infected with *P. berghei* into an atmosphere sufficiently deficient in oxygen will result in a higher grade of infection than is observed in rats in normal room atmosphere. An antibody has been demonstrated in this rodent malaria,[8] and it is possible that the prevention of the formation of this antibody has been effected. However, the rapidly reactivated infection in rats

7. Thurston, J. P. 1953, *Plasmodium berghei*. Exper. Parasitol. **2**: 311–332.

8. Vargues, R. 1951, Etude serologique de l'infection experimentale du rat blanc par *Plasmodium berghei:* disparition d'alexine. Presence d'anticorps fixant le complement. Compt. rend. Soc. de biol. **145**: 1134–1136.

with a declining parasitism must be then a prevention of the effective action of the formed antibody. This twofold explanation cannot be established or eliminated by the evidence presented in this work. We believe that there occurs a functional depression of the reticulo-endothelial system with subsequently lessened capacity for removal of these circulating malarial organisms, since an increase in the grade of infection developed when hypoxia was induced at various periods in the course of infection.

It is of interest to note that rats at the peak of their infection are immediately affected when they are exposed to an atmospheric deficiency of oxygen. The rates of mortality of these rats were unusually high. Gingrich[9] reported that an injection of foreign erythrocytes, which was employed to block the phagocytic system, caused a sustained level of parasitemia when this was done at the time of, or after, the crisis in avian malaria. He found little or no effect by blocking the reticulo-endothelial system with foreign cells, if this were done during or before the acute rise of infection, and interpreted this to be evidence that phagocytosis is not an important factor in natural immunity but comes into play only in the acquired immunity to avian malaria. The untoward effects of a reduced tension of oxygen, which we have observed in avian as well as in rodent malaria, are producible during the rise of infection, at the crisis, or even during the fall of parasitemia, and we interpret these observed effects to be most likely due to an inhibition of the same defensive mechanism of the host.

9. Gingrich, W. D. 1941, The role of phagocytosis in natural and acquired immunity in avian malaria. J. Infect. Dis. **68**: 37–45.

The effects which we have observed of dual infections of *P. berghei* and *T. lewisi* further emphasize that a common factor in the hosts is responsible for the physiological defense against these different protozoal organisms. In these studies with intercurrent infections of *P. berghei* and *T. lewisi* the malarial infection seemed to be more enhanced than that of the flagellate. However, the parasitemic levels of both infections were increased, and hence one cannot say explicitly whether either or both of the two infections proved to be the cause of death of the animals that died under these conditions.

SUMMARY

1. Continued exposure of rats to simulated altitudes of 19,000 to 20,000 feet, beginning at the time of inoculation, shortens the time for the appearance of the organisms of *Plasmodium berghei* in the peripheral blood, when compared to the time required under normal atmospheric conditions. The resultant infection has an enhanced grade of parasitemia as well as an increased mortality rate.

2. Rats infected with *P. berghei* are similarly more severely affected if they are placed in an atmosphere of low concentration of oxygen at the time at which they are at the peak of their infection.

3. Rats which have nearly recovered from an infection with *P. berghei* will respond to induced hypoxia with a secondary rise in parasitemia and a higher percentage mortality.

4. Intercurrent infections of *P. berghei* and *Trypanosoma lewisi* have the effect of significantly increasing concurrently the grade of infection of each of the organisms resulting in a higher mortality rate than in instances of either of the infections alone.

HOST-PARASITE RELATIONSHIP IN EXPERIMENTAL PERTUSSIS

1. HISTAMINE-SENSITIZING AND PROTECTIVE ACTIVITIES OF PERTUSSIS VACCINE IN MICE

CHARLES W. FISHEL

From the Department of Microbiology, The University of Chicago, Chicago 37, Illinois

In recent years the attention of many investigators has been directed to the study of various biochemical changes produced in the host's metabolic systems following parasitic invasion, and with subsequent attempts to reduce to a biochemical basis the conflict between the host and the parasite.

In 1948, Parfentjev and Goodline[1] reported that the natural resistance of the white mouse to histamine was profoundly lowered following the inoculation of *Hemophilus* (*Bordetella*)[2] *pertussis*. This phenomenon appeared to be particularly well suited for the investigation of the relationship between altered host reactivity and the pathogenesis of an infectious disease. First, it is a specific reaction both with respect to the host and to the parasite. The mouse is the only experimental animal in which this phenomenon has been demonstrated and, even in this host, there occur sex[3] and strain[4] differences in developed histamine hyper-reactivity. The pertussis microorganism must be in the smooth, phase 1 form[5] and is unique with respect to degree[6] in its ability to elicit this histamine response of the host. Secondly, it has been shown that histamine hyper-reactivity can be induced by either the live, virulent[7] or the killed microorganisms. Thus, the host changes can be studied both in the presence and in the absence of active infection. Thirdly, the phenomenon may be of practical significance, possibly involved in the immunization side effects; there have been at least 60 well authenticated cases of encephalopathy following the usual immunization procedures.[8–10] There may also be a correlation between this decrease in resistance of the mouse to histamine and the paroxysmal stage of the disease process in human infection.

The following reports revolve around the problem of the relationship between the pertussis-induced histamine hyper-reactivity and the immunity or pathogenesis of the disease. It is proposed to use the term "hyper-reactivity" in place of "hypersensitivity" for, as will be demonstrated, this state is not dependent upon antibody production, at least of the protective type. This problem may be approached in a variety of ways. In the present studies, there has been no

Received for publication February 22, 1956.

This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Appreciation is due to the Eli Lilly Company, Indianapolis, Indiana for supplying the vaccine used in these studies, and to Dr. L. O. Jacobson of the Argonne Hospital, University of Chicago, for the facilities used in the X-irradiation experiments.

* Mr. and Mrs. Frank G. Logan Fellow in Microbiology. Present address: Dept. of Medical Microbiology, Stanford University, Stanford, California.

1. Parfentjev, I. A. and Goodline, A. 1948, J. Pharmacol. & Exper. Therap. **92**: 411–413.
2. Moreno-Lopez, M. 1952, Microbiol. Espanola, **5**: 177–181.
3. Pittman, M. 1951, J. Infect. Dis. **89**: 296–299.
4. Munoz, J. and Schuchardt, L. F. 1953, J. Allergy **24**: 330–334.
5. Halpern, B. N. and Roux, J. 1949, Compt. rend. Soc. de biol. **143**: 923–927.
6. Kind, L. S. 1951, J. Immunol. **70**: 411–421.
7. Pittman, M. 1951, Proc. Soc. Exper. Biol. & Med. **77**: 70–74.
8. Byers, R. K. and Moll, F. C. 1949, Pediatrics, **1**: 437–440.
9. Globus, J. H. and Kahn, J. L. 1949, J.A.M.A. **141**: 507–509.
10. Toomey, J. A. 1949, J.A.M.A. **139**: 448–450.

attempt to fractionate the bacteria into endotoxin and other cell substances differentiable by serological and pharmacological methods. Rather, attention has been directed toward an investigation of the host response. Two hypotheses were formulated: (1) the histamine hyper-reactivity of the mouse following the intraperitoneal inoculation of pertussis vaccine is the result of a direct action of a bacterial substance or substances upon the host's tissue and/or metabolic processes; or (2) this effect is indirect, mediated through the immune responses of the host. If the first hypothesis were operative, interference of the host's metabolic processes could possibly result in the accumulation or disappearance of low molecular weight substances such as the amino acids. Therefore, it was proposed that chromatographic analysis of various tissue extracts following immunization or infection could be useful in the establishment of metabolic changes, if any, and of the possible relationship between histamine hyper-reactivity and these alterations. The results of this chromatographic study will be presented in a future report.

The results of investigations based on the 2nd hypothesis will be presented in the following report. It was of primary concern to establish the independence of the histamine hyper-reactive state and conferred protection. This was achieved in immunized mice who were resistant to intracerebral challenge inoculation of *H. pertussis*, and in mice following a suppression of the immune responses by preimmunization total body X irradiation. Evidence will be presented which demonstrates that the lowering of the resistance of the mouse to histamine following immunization with pertussis vaccine is, most probably, the result of a direct action of a bacterial substance, and is not mediated through the immune responses of the host.

MATERIALS AND METHODS

Mice.—White Swiss, female mice of a pen bred strain, obtained from Porton-Woods, Inc. Wheaton, Illinois were used in all experiments. At the beginning of each experimental period, they were approximately 4 to 5 weeks old with and average weight of 14 to 16 g. So far as susceptibility to *H. pertussis* is concerned, these animals were highly uniform.

Vaccine.—Commercially prepared (Eli Lilly Company) fluid, V-1035 vaccine, was used in all immunization experiments. This vaccine consisted of an unwashed saline suspension of the microorganisms, killed with 0.1% formalin and preserved with merthiolate. As dispensed, this vaccine contained 50×10^9 organisms per ml.

H. pertussis.—The standard challenge strain 18–323 was maintained in the lyophilized state and, previous to each experiment, was cultured on Bordet-Gengou medium for 48 hours and then subcultured to either Bordet-Gengou or charcoal[11] medium for 24 hours. Inocula prepared from this subculture were uniformly in the smooth, phase 1 state of maximal virulence and immunogenic potency.

Aerosol induced infection.—The cloud chamber utilized for the induction of infection was that described by Weiss and Segeler[12] and by Gogolak.[13] The only alteration was to adapt the exhaust system which operated the sampling impingers under ½ atmosphere pressure to function without the subsequent interference with hygrometer readings. Experimental studies of the effect of humidity on particle size and nominal recovery of viable organisms indicated that, with salt-free veal infusion broth as the suspending medium, humidities below 35% did not play a significant role on the nominal recovery but were important in the determination of particle size as measured by the Cascade impactor.[14] It has been demonstrated by Sonkin[15] that lower respiratory infections following the inhalation of streptococci were due mainly to particles smaller than 1.8 μ. VanWijk and Patterson[16] have shown

11. Ensminger, P. W., Culbertson, C. G. and Powell, H. M. 1953, J. Infect. Dis. **93**: 266–268.
12. Weiss, E. and Segeler, J. C. 1952, J. Infect. Dis. **90**: 13–20.
13. Gogolak, F. M. 1953, J. Infect. Dis. **92**: 240–272.
14. Sonkin, L. S. 1946, J. Ind. Hyg. Toxicol. **28**: 269–272.
15. Sonkin, L. S. 1951, Am. J. Hyg. **53**: 337–354.
16. VanWijk, A. M. and Patterson, H. W. 1940, J. Ind. Hyg. Toxicol. **22**: 31–33.

that retention of particulate matter in the respiratory tract was approximately proportional to the square root of the particle diameter and that a critical stage occurred between 0.2 and 0.4 μ where the particle was retained the least. Goldberg and Leif[17] studied the retention of *Pasteurella pestis* in the lungs of mice and concluded that approximately 30% of the calculated dose was retained when the particle size was 1.0 μ in diameter. Preliminary experiments with the Cascade impactor demonstrated that, with humidities of 18 to 28%, the particle size ranged from 0.7 to 4.8 μ with the majority being of the order of 1.0 μ.

From the above data it was concluded that, for maximum recovery of viable *H. pertussis* cells and optimum particle size for retention, the following conditions be applied in all aerosol experiments:

1. Flow through the chamber, 50 liters of air per minute.
2. Relative humidity, 18 to 28%.
3. Air pressure through atomizer, 10 p.s.i.
4. Fluid flow of atomizer, 0.5 ml per minute.
5. Pressure inside the chamber, -1 to -2 inches of water.

Impinger values indicated that, under the above conditions, the nominal recovery of viable *H. pertussis* cells was 45.8%. To calculate the dosage of the aerosol exposed mice, Goldberg and Leif's value of 30% retention was applied to the above value. In this manner, the actual dose was 13.7% of the calculated bacterial content per ml of air in the chamber.

The respiratory volume of the mouse was calculated as 1.25 ml per minute per gram of body weight.[18]

The culture for the aerosol experiments was prepared by growing the cells from the lyophilysed state for 48 hours on Bordet-Gengou agar, the cells suspended in saline and transferred to 400 ml of the charcoal medium without the addition of agar. This was placed on a shaker, agitated sufficiently to keep the charcoal in suspension and incubated at 37 C for 24 hours. The culture was filtered through cotton, centrifuged at 4000 $\times$g for 40 minutes at 0 C. The sedimented cells were resuspended in salt-free veal infusion broth to one-tenth of the original volume. The mice were exposed in individual open wire cages with the maximum number in any single operation being 120. Viable cell counts of the reservoir material before and after atomization indicated that the dosage was constant during the exposure period.

Intracerebral inoculation.—The procedure for the intracerebral inoculation of mice was identical with that as recommended by the National Institutes of Health[19] for challenge in the testing of the potency of pertussis vaccine. The LD_{50} for the mice used in these investigations was found to be approximately 625 viable *H. pertussis* cells. Therefore, this strain of mice was adequately susceptible to infection induced via this route. The 1.0×10^4 dilution of the culture contained in the range of 25 to 100 LD_{50}'s; if a faster death rate was desirable, the 10^3 dilution was utilized. The mice were held for 14 days after the challenge inoculation unless noted otherwise.

There were no visible signs of infection during the first 3 days following the intracerebral inoculation of 25 to 175 LD_{50} viable cells. The first symptoms to develop were inactivity, ruffled fur and "humpiness." The next stage was characterized by paralysis of the hind quarters followed by a general loss of muscular control. In the final stages before death, the head appeared to be greatly enlarged due to edema. The animal was very hunched, unable to stand, and with muscular control completely absent. Autopsy revealed serious brain damage; the consistency of the tissue was so altered that it was quite difficult to remove the brain in toto from the skull.

Immunization.—The routine procedures[19] for the intraperitoneal inoculation of pertussis vaccine were followed and, where it was necessary to vary the number of cells in the dose, dilutions were made in physiological saline in order to maintain a constant inoculation volume. This strain of mice developed hyper-reactivity to histamine which was maximal 5 days following immunization and this reactivity was directly proportional to the number of cells used in the immunizing dose. These mice were also relatively resistant to the toxic effects of high doses of vaccine for, in one experiment, 140 mice were immunized with 2.5×10^{10} cells with only 4 deaths occurring due to the toxicity of the vaccine.

Agglutination tests.—The antigen was prepared by suspending the 24-hour growth on Bordet-Gengou medium in 0.85% saline and adding formalin in a final concentration of 0.2%. This standard antigen contained 1.0×10^{10} cells per ml.

To 0.2 ml of the serum dilution was added 0.3 ml of the antigen, the tubes were shaken for 5

17. Goldberg, L. J. and Leif, W. R. 1950, Science, **112**: 299–301.
18. Rosebury, Theodor 1947, Experimental Airborne Infection, Baltimore, Williams & Wilkins Co.
19. Circular, 1952, National Inst. of Health: Min. Req., pertussis vaccine, 1st Revision, Bethesda, Md.

minutes and placed in a water bath at 56° C for 3 hours. After incubation, the tubes were placed in the refrigerator overnight and the results read the following morning.

Sensitivity to histamine.—The histamine LD_{50} was determined by the intraperitoneal injection of histamine diphosphate in sterile distilled water. Serial dilutions to 2^n were prepared from a standard solution containing 30 mg of histamine base per ml and the challenge doses were contained in 0.5 ml volume. The LD_{50} was expressed as the amount of histamine base required to kill 50% of the animals as calculated by the moving average method of Thompson,[20] interpolation by Weil.[21] Generally, 4 levels of challenge, 10 mice per level, were used. Results could be read with accuracy 2 hours after the inoculation of histamine.

X irradiation.—The mice were irradiated in small, ventilated plastic tubes arranged in a circle on a rotating table. The X-ray machine was operated to deliver 59.7 r per minute with 0.25 mm copper and 1.0 mm aluminum filters. The distance from the target to the center of the mouse was 70.5 cm. Preliminary experiments indicated that a dose of 600 r would result in approximately 30% survival 14 days after irradiation (LD_{70}[14]) with no deaths occurring for the first 6 days and also indicated that the immune responses of the host were greatly suppressed at this dosage level. Therefore, a dose of 600 r was used in all X-irradiation experiments.

EXPERIMENTS AND RESULTS

Effects of the number of cells in the immunizing dose.—It has been demonstrated by Munoz and Schuchardt[4] that, in general, the decrease in resistance of mice to histamine following immunization with pertussis vaccine was directly proportional to the number of cells used in the inoculum. This observation was confirmed for the particular strain of mice used in the present studies, as shown in table 1.

Four groups of mice, 40 per group, were immunized with various doses of vaccine and challenged with histamine 5 days later. Both injections were made intraperitoneally. Data in table 1 show that individuals immunized with 2.5 $\times 10^{10}$ cells were approximately 60 times more sensitive to histamine than were the nonimmunized controls and this increase was, within experimental limits, directly proportional to the number of cells in the immunizing dose.

TABLE 1.—*Sensitivity of mice to histamine 5 days after immunization with various dosages of pertussis vaccine.*

Number of cells in the immunizing dose ($\times 10^{10}$)	LD_{50}: mg of histamine base	% confidence interval
2.50	0.246	0.149– 0.418
0.70	4.350	2.230– 8.540
0.20	12.500	9.670–16.900
0.08	14.900	10.600–20.900
None	14.900	10.600–20.900

It was postulated that if the protective and sensitizing properties of the vaccine were identical, then the protective activity, i.e., the ability to elicit protective antibody response, of a vaccine should decline proportionally with a decrease in the sensitizing property in mice which were immunized with various and decreasing numbers of cells. To test this postulate, a series of 3 experiments were conducted. One-hundred and fifty mice were divided into 5 equal groups. One group was immunized with 2.5×10^{10} *H. pertussis* cells, a 2nd group with 0.7×10^{10}, a 3rd group with 0.2×10^{10}, a 4th with 0.08×10^{10}, and the 5th group was held as the nonimmunized controls. Five days after the inoculation of the vaccine, all groups were challenged intracerebrally with 25 LD_{50}'s of the microorganism. The time between immunization and challenge did not conform to the interval recommended by the National Institutes of Health,[19] so that the observed immunity is not directly comparable with that obtained by the standard method. The results of these experiments are given in figures 1 and 2.

Figure 1 demonstrates that not only did the highest dose of vaccine (2.5×10^{10} cells) confer but slight protection within the 14-day experimental period

20. Thompson, W. R. 1947, Bact. Rev. **11**: 115–120.
21. Weil, C. S. 1952, Biometrics, **8**: 249–263.

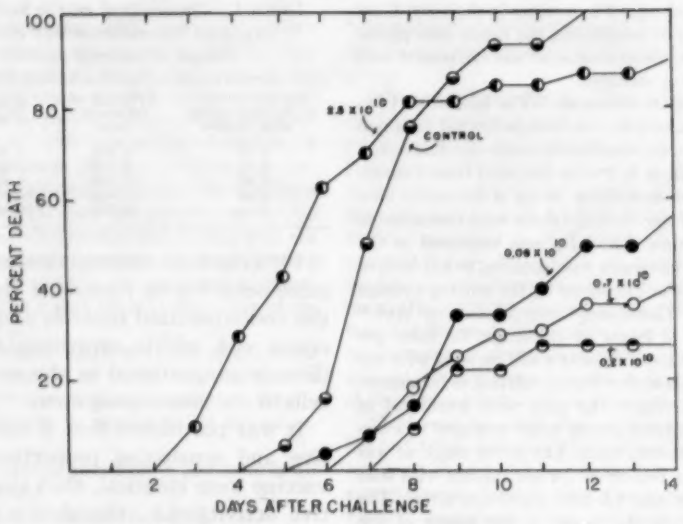


FIG. 1.—Intracerebral challenge of pertussis vaccinated mice. Four groups of mice were immunized with various numbers of cells and challenged 5 days later with 25 LD_{50}'s of *H. pertussis*. Another group similarly challenged served as nonimmunized controls. The percent of deaths was recorded for 14 days following the challenge inoculum.

with less than 10% survival but also, for the first 6 days after challenge, mice immunized with this dose were more susceptible to the challenge inoculum than were nonimmunized controls. A group comparison on the 6th day after challenge shows that approximately 84% of the controls were alive with only a 37% survival of those immunized with the highest dose of vaccine. This "optimal dosage" phenomenon will be discussed in more detail later in this report.

Figure 2 is a representation of the degree of protection conferred by the various doses of vaccine 14 days after the challenge inoculation, with the histamine LD_{50} determined on the 5th day following immunization. These data indicate that: (1) in confirmation of previous results, the resistance to histamine after immunization with pertussis vaccine was directly proportional to the number of cells in the immunizing dose; and (2) the degree of protection, within limits, was neither correlated with the number of cells in the immunizing dose nor to the development of histamine hyper-reactivity. At the extremes, mice vaccinated with 0.08×10^{10} cells failed to develop a significant change in resistance to histamine and were comparable to the nonimmunized controls. However, 40% of the immunized mice at this dosage level were protected against the challenge inoculation. At the other extreme, those immunized with 2.5×10^{10} cells were extremely sensitive to histamine at the 5-day period, but only 5% were protected against the same challenge dose.

These results support the view that the histamine-sensitizing and protective phenomena as manifested in immunized mice are separable. However, these experiments do not rule out the possibility that the histamine hyper-reactivity is, at least in part, based upon antibody production. Therefore, the effect of suppression of antibody formation by

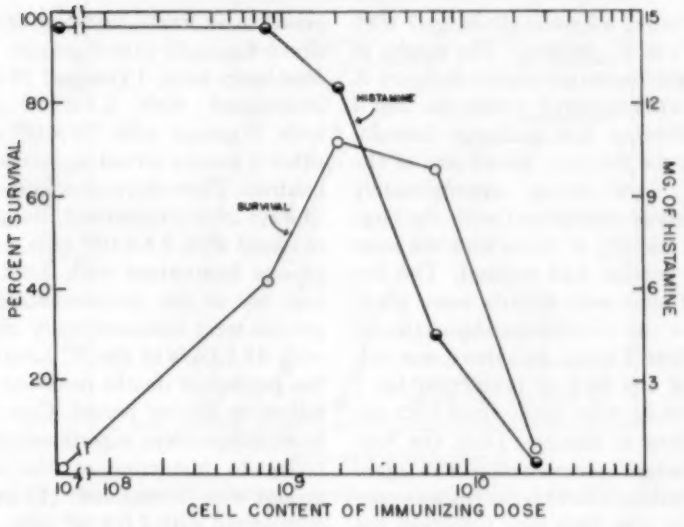


FIG. 2.—Comparison of conferred protection and developed hyper-reactivity to histamine. Histamine LD_{50} determined 5 days after vaccination, and the survival from figure 1, 14 days after challenge.

total body X irradiation was next studied.

Effect of total body X irradiation.—It was postulated that, if the sensitizing and protective phenomena were identical, then suppression of the immune responses of the host should also result in the suppression of histamine-sensitizing property.

A number of reports in literature (reviewed by Taliaferro and Taliaferro[22] and by Dixon[23]) have shown that preinoculation total body X irradiation, under certain circumstances, is effective in suppressing or eliminating the active formation of antibody.

Preliminary experiments demonstrated that preimmunization X irradiation (600r) did not alter the time element involved for the development of the histamine hyper-reactivity. The maximum sensitivity was attained on the 5th day following immunization, regardless of the pre-exposure to X irradiation.

In the following experiments, a 3-day period intervened between immunization and the intracerebral challenge with *H. pertussis*. This period was chosen for several reasons: (1) it was desirable to complete the experiment before significant radiation mortality had occurred; (2) it has been reported by Evans and Perkins[24] that 3 days after the injection of pertussis vaccine, 70% of the mice were resistant to challenge inoculations of from 75 to 125 LD_{50}'s; and (3) it is within this period that the development of histamine hyper-reactivity first appears.

For nonirradiated control purposes, 105 mice were divided into 3 equal groups. One group of 35 was immunized with 2.5×10^{10} cells, a 2nd group with 0.7×10^{10} cells, with the 3rd group serving as the nonimmunized controls. Three days after the immunization of

22. Taliaferro, W. H. and Taliaferro, L. G. 1951, J. Immunol. **66**: 181–212.

23. Dixon, F. J. Talmage, D. W. and Maurer, P. H. 1952, J. Immunol. **68**: 693–701.

24. Evans, D. G. and Perkins, F. T. 1954, Brit. J. Exper. Path. **35**: 322–330.

the 2 groups, all were challenged with 63 LD_{50}'s of *H. pertussis*. The results of these experiments are shown in figure 3.

No deaths occurred within the first 4 days following the challenge inoculation. On the 5th day, before any of the controls were dying, approximately 14% of those immunized with the largest dose and 6% of those with the least dose of vaccine had expired. The immunized mice were slightly more sensitive than the controls to the challenge for the first 5 days, and there was evidence for the lack of protection for 7 days in those mice immunized with the greater dose of vaccine. Thus, the "optimal dosage" phenomenon was again demonstrable. The experiment was concluded on the 10th day following the challenge inoculation, at which time 80% of the mice which had received the largest dose of vaccine, and 50% of the other vaccinated group had expired.

The effect of preimmunization total body X irradiation was next studied. One-hundred and fifty mice were exposed to an irradiation dose of 600r and divided equally into 5 groups. Twenty-four hours later, 1 group of 30 mice was immunized with 2.5×10^{10} pertussis cells, 2 groups with 7.0×10^{9} cells, the other 2 groups served as nonimmunized controls. Three days after immunization (4 days after irradiation), the group immunized with 2.5×10^{10} cells, one of the groups immunized with 7.0×10^{9} cells and one of the nonimmunized control groups were intracerebrally challenged with 63 LD_{50}'s of the *H. pertussis* with the percent of deaths recorded over the following 10-day period. Concurrently, in an independent experiment, the sensitivity to histamine of the following groups was determined: (1) irradiated, immunized with 7.0×10^{9} cells; (2) nonirradiated, immunized with 7.0×10^{9} cells; and (3) irradiated only. This determination was made on the 5th day following immunization or 6 days following X irradiation. The results of these experiments are shown in figure 4.

A group comparison on the 4th day

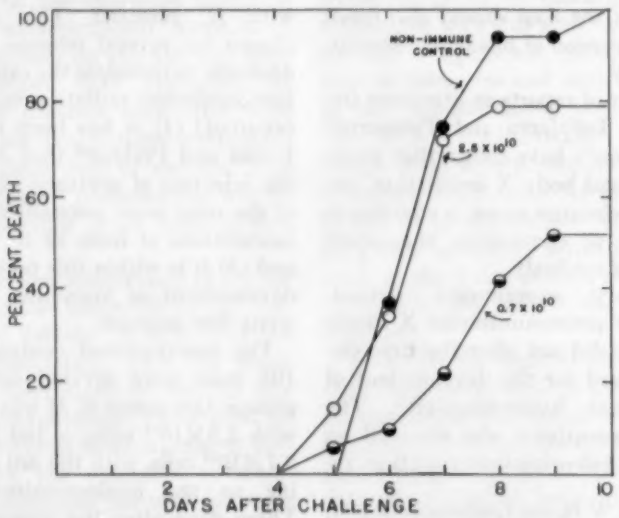


FIG. 3.—Percent mortality resulting from the intracerebral challenge with 63 LD_{50}'s of *H. pertussis* of non- and immunized mice. Three days intervened between the inoculation of the 2 dosages of pertussis vaccine and the challenge inoculation.

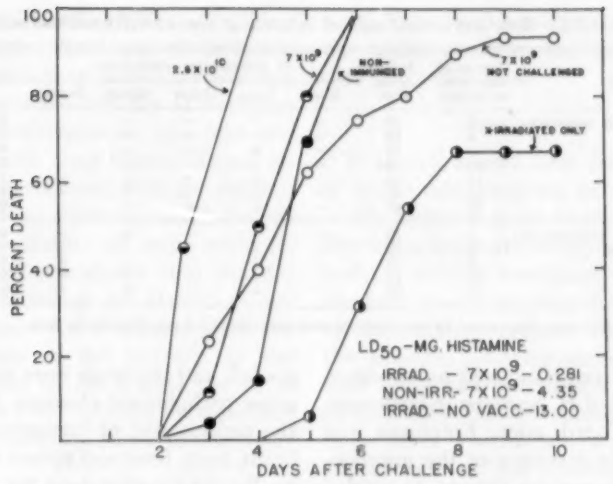


FIG. 4.—Effect of preimmunization X-irradiation. Explanation of the figure in text.

after challenge shows that all of the mice which had been immunized with the highest dose of vaccine were dead, as were approximately 50% of those immunized with 7.0×10^9 cells and challenged, and 40% of those immunized with the latter dose of vaccine but not challenged. At this same interval, only 15% of the nonimmunized controls were dead, and with no deaths due to the effect of irradiation alone. Examination of the histamine LD_{50} shows that exposure to this dose of irradiation without subsequent immunization did not alter the normal resistance of the mouse to histamine, but irradiation plus immunization rendered the animal even more sensitive than does immunization alone. It is evident that: (1) immunization did not confer any degree of protection and actually sensitized the irradiated mice to the challenge inoculation; and (2) irradiation decreased the resistance of the animal to the toxic effects of the vaccine. From the data presented in figures 3 and 4, it would seem evident that the immune responses of the host animal were greatly suppressed following total body X irradiation at this dosage level. On the 4th day after challenge, before any deaths had occurred in the nonirradiated group, the above results had taken place in those exposed to X irradiation. The 5th day after challenge magnifies the difference in response of these 2 groups.

These results indicate that the immune responses, of which antibody production seems to play a major role, were greatly suppressed by preimmunization X irradiation; at the same time, these mice were approximately 20 times more sensitive to histamine than were nonirradiated controls with both groups receiving identical immunizing dosages. Therefore, it would seem that the histamine-sensitizing and protective activities of pertussis vaccine are separable in mice in which no antibody formation or other evidence of acquired immunity is demonstrable. It appears that some chemical fraction of *H. pertussis* must have the ability to react directly with some tissue component or metabolic system of the mouse to produce the hyper-reactive state.

Aerosol induced infection.—The purpose of this experiment was to follow the

TABLE 2.—*Results of aerosol induced infection of non- and X-irradiated mice.*

Group	Days after exposure to aerosol	Agglutinin titer	*H. pertussis* recovered from:					Deaths	Sensitivity to histamine*
			Brain	Lung	Liver	Spleen	Serum		
1. Normal controls nonirradiated	1	10	–	–	–	–	–	0/100	0/5
	4	10	–	–	–	–	–	0/85	0/5
	7	10	–	+	–	–	–	0/70	0/5
	10	20	–	+	–	–	–	0/55	2/5
	15	80	–	+	–	–	–	1/40	3/5
	20	40	–	+	–	–	–	3/24	5/5
	30	10	–	+	–	–	–	0/9	5/5
2. 600r 24 hours previous to aerosol exposure	2	10	–	–	–	–	–	0/100	0/5
	4	10	–	+	–	–	–	0/85	0/5
	7	10	–	+	–	–	+	5/70	2/5
	10	10	–	+	–	+	+	20/55	5/5
	15	10	–	+	–	+	+	40/40	

* Sensitivity to histamine determined by the intraperitoneal injection of 2.0 mg of histamine base.

course of infection induced by exposure to an aerosol of *H. pertussis* in both non- and X-irradiated mice. Emphasis was placed on the recovery of the microorganism from various tissues, to agglutinin production, to the type and the gross pathological pattern of the infectious process, and to the development of histamine hyper-reactivity induced in this manner.

Two-hundred mice, of which 100 had been exposed to an irradiation dose of 600r 24 hours before, were exposed to an aerosol of *H. pertussis* in the cloud chamber. Each mouse received a calculated dose of 3.3×10^8 viable cells. At various intervals, groups of 15 mice were removed from each pool, 5 of which were challenged intraperitoneally with 2.0 mg of histamine base. The remaining 10 were sacrificed, the serums collected and pooled, and the lungs were examined for gross pathological changes. Aliquots of the serums and of homogenates of the brain, lung, liver and spleen were plated on Bordet-Gengou agar for determination of recovery of the microorganism. The results of this experiment are given in tables 2 and 3.

These results show that in the nonirradiated (group 1) mice, the agglutinin titer rose to a maximum of 1:80 by the 15th day and gradually decreased thereafter to less than 1:10 by the 30th day. The microorganism was not recoverable from any of the examined tissues or serums until the 7th day after exposure and then only from the lung tissue. This lasted for the duration of the experimental period. Mortality, as the result of this particular infection, was very low, and the extreme sensitivity to histamine

TABLE 3.—*Gross lung pathology of non- and X-irradiated mice following aerosol induced infection.**

Group	Days after aerosol exposure						
	2	4	7	10	15	20	30
1. Normal controls—nonirradiated	All normal	All normal	6–normal 1–D† 2–1 plus 1–3 plus	2–normal 8–D	2–normal 3–1 plus 5–D	4–normal 2–1 plus 3–2 plus 1–4 plus	4–D 2–1 plus 3–3 plus 1–4 plus
2. 600r 24 hours previous to aerosol exposure	All normal	5–normal 4–D 1–3 plus	‡ 3–normal 4–D 2–1 plus 1–4 plus	2–normal 2–D 3–2 plus 1–3 plus 2–4 plus	All Dead	§	

* Lung consolidation reported of those mice sacrificed at the various intervals unless otherwise noted. Consolidation reported as 1, 2, 3, or 4- plus dependent upon the extent of lung involvement.

† D indicates a discoloration of the lung tissue as previously reported by Pittman.

‡ Post-mortem examination of the lungs of the 5 mice which died as a result of infection at this time interval indicated that all 5 were of 4- plus consolidation.

§ The lungs of ten dead exhibited 3- and 4- plus consolidation.

was not manifested until the 20th day after exposure. Evidence of infection based on gross lung pathology was not apparent until the 7th day at the same time the microorganism was first isolable from the lung tissue. These results are in agreement with the original observations of Pittman[7] after the intranasal inoculation of mice with *H. pertussis*. She postulated that the decrease in resistance to histamine appeared to have some relation with the multiplication of the bacteria in the lungs and not to the lesions per se as both lesions and bacteria were present before the maximum period of reactivity developed.

In the irradiated mice (group 2), the disease picture was changed into that of an acute, fulminating type of infection. The agglutinin titer failed to rise above 1:10, the bacteria were recoverable from the lung tissue on the 4th day and also from the sera and spleen on the 7th and 10th days respectively. A high percentage of the deaths occurring on the 10th day might possibly be due to the effects of irradiation. However, the 100% mortality on the 15th day is significant. Gross pathological changes in the lung tissue occurred on the 4th day, and the histamine hyper-reactivity was developed 10 days before that of group 1.

The results of this experiment also demonstrate that the histamine hyper-reactive state develops in both non- and X-irradiated groups. This occurs despite the fact the immune responses of the latter group were almost completely suppressed as shown by the absence of rise in agglutinin titer, the lack of localization of the bacteria, the increase in mortality, and a more rapid development of gross pathological changes in the lungs. This observation provides further evidence for proposing that the histamine hyper-reactivity developed after the inoculation of *H. pertussis* is a separable response and is not dependent upon the immune mechanics of this particular host.

DISCUSSION

It is well known that certain strains of white mice respond to an injection of *H. pertussis* in at least 2 ways: (1) develop a hyper-reactivity to histamine, and (2) exhibit resistance to an intracerebral challenge inoculation of the virulent microorganism. The results of the present investigation demonstrate that these two reactions are separable as shown by: (1) the resistance to histamine after vaccination was inversely proportional to the number of cells in the immunizing dose, but the degree of protection was, within limits, neither correlated with the number of cells in the immunizing dose nor to the development of histamine hyper-reactivity; (2) the immune responses of irradiated mice following vaccination or aerosol-induced infection were greatly suppressed. At the same time, mice in the former group were approximately 20 times more sensitive to histamine than were nonirradiated controls, and those of the latter group developed the hyper-reactive condition at a much faster rate than did the nonirradiated controls. It was postulated that some chemical fraction of *H. pertussis* must have the ability to react directly with some tissue component or metabolic system of this host to produce the sensitive state. A possible explanation of the increase in histamine sensitization induced by preimmunization X irradiation above that induced by immunization alone might therefore be that irradiation alters the tissues or renders some particular enzyme system more susceptilbe to the sensitizing ability of the pertussis microorganism. In this report, the assumption has been made that all forms of antibody production have been sup-

pressed by exposure of the mice to total body X irradiation of 600 r. Although the weight of the presented evidence indicates that this assumption is correct, the possibility of radioresistant antibody formation is recognized. With the dosages of irradiation used, this possibility seems quite remote but does prohibit the definite exclusion of antibody involvement in the production of the histamine hyper-reactive state.

As far as can be determined, this is the first reported study of pertussis infection in mice induced by an aerosol. Although the results are in complete agreement with those reported by Pittman[7] following intranasal inoculation, it is of significance that fewer bacteria were required to produce the same gross pathology and reduction of resistance to histamine when the cloud chamber method was used. This may be accounted for by the uniformity of infection, abolition of the effects of anaesthesia, and the reduction of the amount of extraneous material introduced into the alveolar tissue. The experimental results of the aerosol investigations indicate that preinoculation X irradiation may provide a new approach to the problem of infecting a resistant host. The infectious process produced by this particular microorganism changes from that of a quiescent, low-mortality type in the nonirradiated group into an acute, fulminating disease as the result of a reduction or suppression of the immune responses of the host by X irradiation. It was demonstrated that agglutinin production was suppressed, the microorganism was recoverable from other than lung tissue, and that mortality was increased in the latter group. However, there are many other effects of irradiation damage which may also play significant roles in the phenomenon of increased susceptibility to infection.

The question of the inactivation, by *H. pertussis*, of an enzyme concerned in the detoxification of histamine is also debatable. Kind and Woods[25] reported that lung tissue from pertussis inoculated, histamine sensitive mice had a reduced capacity to destroy this amine. The present irradiation studies do not discount this theory for there are many enzymes which are resistant to the inactivating effects of X ray. However, it is believed that this question requires further study before definite conclusions may be established.

During the course of the present studies, it became evident that there was an optimal dosage of vaccine which would confer maximum protection to mice, at least in the early developmental stages of acquired immunity, against intracerebral challenge inoculations. The better protection conferred by a low dose than with a high dose of vaccine has been noted before, especially following alum precipitation. In the test of alum precipitated vaccines for immunogenic potency, a smaller number of pertussis cells are used in the immunizing dose than in the test of the fluid type. The period between vaccination and challenge is 4 days longer with the precipitated vaccine. There would appear to be at least 2 explanations of this "optimal dosage" phenomenon. First, that it is due to a toxic factor present in the vaccine and the phenomenon is due to a dilution of this toxic factor. It is also possible that this factor is the sensitizing agent, for the toxicity of the vaccine is magnified above the normal by preimmunization X irradiation, and, at the same time, such mice are more susceptible to histamine than are controls.

The second explanation would appear to be that of a manifestation of "im-

25. Kind, L. S. and Woods, E. F. 1953, Proc. Soc. Exper. Biol. & Med. **84**: 601–603.

munological paralysis." Felton[26] demonstrated that mice injected with relatively large amounts (0.5 mg) of pneumococcal polysaccharide are subsequently incapable of being immunized with the homologous antigen and are more susceptible to pneumococcal infection. Dixon,[27] working with the same system, indicated that any persisting antigen in excess of the amount involved in stimulating antibody production would seem capable of soaking up antibody as rapidly as it is formed, thereby negating the immunological response of the host. It seems possible that this same process is operative in the case of *H. pertussis*, the difference in the length of time in the "paralyzed" state explanable by the difference in persistence of the different antigens in the host.

Whether the immunogenic potency of pertussis vaccine in man, as measured by the effective immunity produced, is subject to similar variability is open to question. If the response in man is analogous to that of the mouse, immunization may result in an increased susceptibility to infection for a short period of time immediately following vaccination.

It is not to be inferred from these results that the sensitizing and protective properties of the pertussis vaccine are two separate and distinct entities. It is possible that both of these states are produced by the same component of the bacterial cell. However, these results have demonstrated that the responses of the host to the entity or entities are separable, with one dependent upon immune mechanisms while the sensitizing is most probably independent of such mechanisms.

26. Felton, L. D. 1926, Bull. Johns Hopkins Hosp. **38**: 33–41.
27. Dixon, F. J., Maurer, P. H. and Weigle, W. O. 1955, J. Immunol. **74**: 188–192.

SUMMARY

The decrease in resistance of the mouse to histamine and the conferred protection against intracerebral challenge following the inoculation of *Hemophilus pertussis* were shown to be separable responses, and that antibodies are probably not involved in the production of the histamine hyper-reactive state. It was postulated that some chemical fraction of this particular microorganism has the ability to react directly with some tissue component and/or metabolic system of the mouse to produce this state.

Total body X irradiation suppressed the immune responses of the host, and thus rendered the animal more susceptible to aerosol induced infection, more susceptible to the toxic factor of the vaccine, and increased in degree the histamine hyper-reactivity developed following immunization with pertussis vaccine.

An "optimum dosage" phenomenon occurred following the immunization of mice with various numbers of pertussis cells and was operative, at least, for the first 5 days following the injection of the vaccine. Better protection against an intracerebral challenge was conferred with a dose of vaccine which did not maximally increase the sensitivity of the mouse to histamine, and a degree of protection was conferred with an amount which did not alter the sensitivity. It was postulated that this phenomenon occurred as a result of either a dilution of a toxic factor present in the vaccine or a manifestation of the paralysis of immune responses.

CROSS INFECTIONS AMONG BRUCELLA INFECTED GUINEA PIGS

G. BRIGGS PHILLIPS, GROVER C. BROADWATER, MORTON REITMAN, AND ROBERT L. ALG

From Fort Detrick, Frederick, Maryland

Cross infection of animals with *Mycobacterium tuberculosis* was reported 26 years ago.[1] More recently, cross infection was observed as a result of aerosol challenge With *Bacillus anthracis*.[2] Although isolation cages or ventilated cage[3,4] are often used for holding experimentally infected animals, cages are usually designed to house two or more animals. Depending upon the design of the experiment, animals having the same or different levels of resistance and challenged with equal or unequal doses may be housed in the same cage. Because of accessory doses received from organisms released by aerosol-contaminated fur, the incidence of cross infection may be higher in animals challenged by the respiratory route. Cross infection in such cases may invalidate experimental results.

In the present investigation, aerosol-exposed guinea pigs were housed communally with unexposed cage-mate controls to detect cross infection.

Received for publication February 27, 1956.

Presented in part at the 55th General Meeting of the Society of American Bacteriologists held in New York City, May 1955.

* Inlet and exhaust air was filtered through 2 layers of PF105 spun glass (Owens-Corning Fiberglass Corp., Toledo, Ohio.)

1. Lurie, M. B. 1930, Air-borne contagion of tuberculosis in an animal room. J. Exper. Med. **5**: 743–751.
2. Druett, H. A., Henderson, D. W., Packman, L. and Peacock, S. 1953, Studies on respiratory infection. I. The influence of particle size on respiratory infection with anthrax spores. J. Hyg. **51**: 359–371.
3. Wedum, A. G. 1953, Bacteriological safety. Am. J. Pub. Health, **43**: 1428–1437.
4. Young, G. A. and Underdahl, N. R. 1953, Isolation units for growing baby pigs without colostrum. Am. J. Vet. Res. **14**: 571–574.

MATERIALS AND METHODS

Guinea pigs (Hartley strain) weighing approximately 350 g each were challenged with *Brucella suis*. Cultures were grown in tryptose broth (Difco) for 72 hours at 37 C. Animals were exposed bodily for 5 minutes in a closed chamber to aerosol produced by a University of Chicago Toxicology Laboratory atomizer.[5] During exposures, the aerosol concentrations in the chamber were determined with liquid impinger samplers. Exposed animals were placed in 9″×10″×18″ solid sided ventilated cages*[3] which were ventilated with filtered air at the rate of 57 liters per minute. Unexposed guinea pigs (cage-mate controls) were placed with exposed animals at certain times and removed as indicated for each experiment. After a holding period of 28 days, each animal was sacrificed, autopsied and samples of the lung, spleen and peribronchial lymph nodes cultured for *Br. suis* on tryptose agar.

EXPERIMENTAL

In the first experiment, 12 groups of 20 guinea pigs each were exposed to aerosol concentrations ranging from 80 to 4500 viable brucella organisms per liter of air. The dose (inhaled organisms per animal) was calculated for each group.[6] The first group inhaled 48 organisms per animal and the last group inhaled 2800 each. Immediately after exposure the guinea pigs were placed in ventilated cages, 5 animals per cage. One cage-mate control was placed in each of 2 cages from each group (each group constituted 20 animals or 4 cages) before exposed animals were placed in the cages. One cage-mate control was placed in each of the 2 re-

5. Rosebury, T. 1947, Experimental Air-Borne Infection, Baltimore, The Williams and Wilkins Company, p. 18.
6. Guyton, A. C. 1947, Measurement of the respiratory volumes of laboratory animals, Am. J. Physiol. **150**: 70–77.

maining cages from each group on different days after the first holding day but not after the 7th holding day. Control cage-mates remained with the exposed animals for all or the remaining part of the 28-day holding period. All animals (exposed or control) were sacrificed on the 28th day.

The 2nd experiment was designed to determine if cross infection could result from contact for 24 hours of cage-mate controls with one aerosol-exposed animal. Five guinea pigs were bodily exposed for 5 minutes to *Br. suis* aerosol of approximately 490 organisms per liter of air giving a calculated dose of 300 inhaled organisms per animal. Each animal was placed in a separate ventilated cage. *One hour after exposure*, 3 cage-mate control guinea pigs were placed in each of the 5 cages. After 24 hours, the control animals were transferred to clean cages which were held in a separate room until autopsy. Six days after exposure (7th holding day) 5 other control animals were placed with 1 exposed animal in each of 2 ventilated cages. After 24 hours the cage-mates and exposed animals were moved to separate open cages until autopsy. All animals were autopsied on their 28th holding day.

During these experiments various unsuccessful attempts were made to recover brucella organisms from the air and surfaces within the cages. Because of the failure of these efforts, further studies were made with spores of *Bacillus subtilis* var. *niger*. Two guinea pigs were exposed bodily to air-borne suspensions of spores (6×10^8 spores per liter of air for 3 minutes) and placed separately in sealed ventilated cages which had been previously sterilized. The cages were placed on a table and ventilated through inlet and outlet spun glass filters at the rate of 57 liters of air per minute. The air exhaust (before filtration) from each cage was sampled for *B. subtilis* spores twice each week-day for 3 weeks, using millipore filters. Filter holders were attached to the water bottle opening in the top of the cage. Each sample was taken for 10 minutes (total 283 liters) while the normal ventilating air was off.

RESULTS

In the first experiment, 93% of the guinea pigs exposed to aerosol were infected with *Br. suis* (table 1). One of the 48 controls was lost by suffocation. The number of infected cage-mate controls was not dependent upon the dose (48

TABLE 1.—*Infection of cage-mate guinea pigs with* Br. suis *by continuous exposure to aerosol-exposed animals.*

Group	Aerosol exposure dose	Number of aerosol exposed guinea pigs infected	Number of cage mates infected	Day on which control guinea pigs were placed in cage with aerosol exposed animals:						
				1	2	3	4	5	6	7
1	48	20/20*	2/4*	+ −		+	−			
2	224	20/20	3/4	+ +		−	+			
3	259	18/20	1/4	+ −				−		−
4	287	19/20	1/4	+ −	−				−	
5	335	19/20	3/4	+ +		+	−			
6	400	18/20	3/4	+ −	+				+	
7	440	16/20	2/4	+ −				+		−
8	524	20/20	1/4	+ −				−		−
9	588	20/20	1/3	+		−	−			
10	1070	17/20	2/4	+ −	−				+	
11	1490	20/20	1/4	+ −				−		−
12	2800	20/20	3/4	+ +	−				+	
Totals		224/240	23/47	15/23	1/4	2/4	1/4	1/4	3/4	0/4

\+ Indicates brucella organisms isolated at autopsy on 28th holding day.
− Indicates no brucella organisms isolated at autopsy.
* $\frac{\text{numerator}}{\text{denominator}} = \frac{\text{infected}}{\text{total}}$.

TABLE 2.—Br. suis *infection of cage-mate control guinea pigs housed 24 hours with aerosol-exposed animals**

Remarks	Number of cages	Number of infected control animals† (infected/total)	Percent of infected control animals
Three cage-mates placed in each ventilated cage with one aerosol-exposed animal at 1 hour after challenge	3	13/15	86
Five cage-mates placed in each ventilated cage with one aerosol-exposed animal on the 7th day post challenge	2	5/9	53

* Whole body exposure to 490 org/1/5 min. All animals became infected.

† Infection determined by recovery of *Br. suis* at necropsy.

to 2800) received by the animals exposed in the aerosol chamber. Time of placement in the cages also had no effect, except that no cross infection was noted in 4 animals placed with exposed animals on the 7th day. For instance, 6 of 12 controls placed with animals receiving 48 to 259 organisms were infected and 6 of 12 controls placed with exposed animals receiving 1070 to 2800 organisms were also infected. Somewhat similarly, 15 of 23 controls placed on the first day with aerosol-exposed animals were infected, but 4 of 8 controls placee on the 5th and 6th day with aerosol-exposed animals were also infected.

The conditions of the 2nd experiment were more critical because the cage-mates were in contact with the exposed guinea pigs for only 24 hours and because 3 or 5 cage-mates were placed with only one exposed animal. Table 2 shows that all 5 of the animals exposed in the aerosol chamber were infected. Among the controls, 13 of the 15 placed in contact with exposed guinea pigs for 24 hours on the 1st day after exposure became infected, as did 5 of 9 placed with the exposed animals for 24 hours on the 7th day. The 10th cage-mate guinea pig was lost by suffocation.

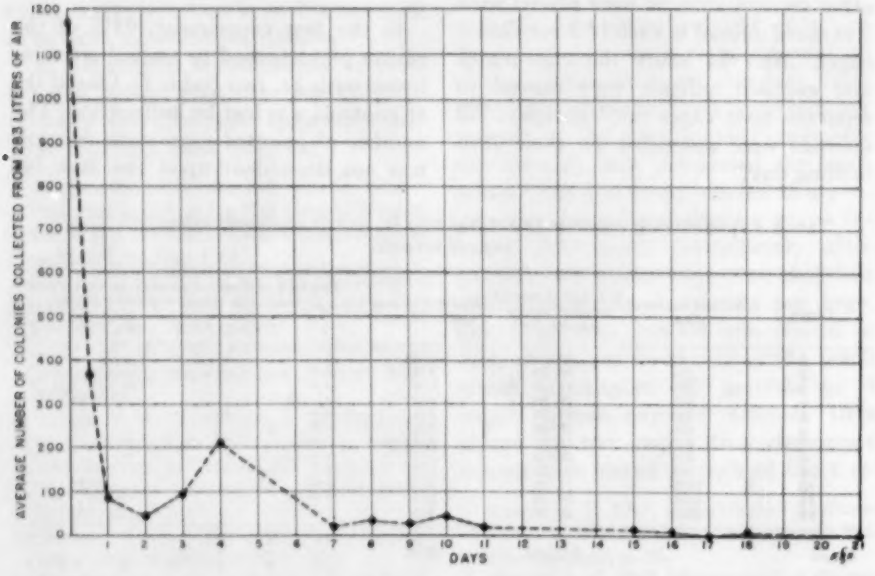


FIG. 1.—Recovery of *B. subtilis* spores from the air of ventilated cages housing aerosol-exposed guinea pigs.

In the experiment in which *B. subtilis* was used as a tracer, few organisms were recovered from the exhaust air of ventilated cages housing aerosol-exposed guinea pigs, considering the large number of organisms in the aerosol to which the animals were exposed. The highest detectable aerosol concentration was approximately 4 spores per liter of air (fig. 1), and spores were recovered up to 18 days. Variations in the day to day samples could be correlated approximately with periods of activity of the guinea pigs.

DISCUSSION

Cross infection in the first experiment may have been caused by brucella from (1) the hair and expired air of the chamber-exposed animals and/or (2) urine and feces. Obviously it would be difficult to separate these sources with any degree of accuracy. Since it is likely that significant numbers of brucella organisms would not appear in the urine and feces of exposed animals within the first 24 hours, it is probable that the cage-mate controls exposed in the second experiment for only the first 24 hours became infected from the hair and expired air rather than from exudates. On the other hand, those control animals which were not put in contact with exposed animals until the 7th holding day may have been infected by organisms from urine and feces, since it is believed that, at a constant ventilation rate of 57 l/min very few of the original contaminating brucella would survive for 7 days on animal hair or on surfaces. However, if a more resistant microorganism is involved (as in the tests with *B. subtilis* spores) it is clear that the original contaminating organisms may cause cross infections for an extended time.

The results obtained in these studies indicate that cross infection should be considered in the design of experiments when animals are challenged by the respiratory route. Further studies must be conducted to determine the importance of cross infection among animals inoculated by other routes as well as the occurrence of cross infection with other infectious organisms.

When highly infectious organisms such as brucella are involved, cross infection among aerosol-exposed animals can probably be eliminated, and thus the validity of the experiment protected, only by housing animals individually in ventilated cages.

SUMMARY

Significant cross infection occurred when normal guinea pigs were placed as controls in ventilated cages with guinea pigs exposed bodily to aerosol of *Brucella suis*. Cross infection was demonstrated when the control animals were housed with aerosol-exposed animals (1) continuously for 28 days, (2) for the first day after exposure only and (3) for the 7th day only.

When guinea pigs were exposed to large numbers of aerosolized *Bacillus subtilis* spores and held in ventilated cages, spores were detected in the air of the cages for as long as 18 days.

THE SUSCEPTIBILITY OF SEVERAL DIFFERENT LABORATORY ANIMALS TO INFECTION WITH *LEPTOSPIRA POMONA*

LEIF M. RINGEN AND WILLIAM OKAZAKI

From the Department of Vertinary Microbiology, College of Veterinary Medicine, State College of Washington, Pullman, Washington

The detection of pathogenic leptospira in contaminated materials is most easily accomplished through the use of laboratory animals. At the present time, guinea pigs and hamsters are the most commonly used animals, both being quite suceptible to leptospiral infection. Borg-Peterson[1] found that guinea pigs and albino mice were susceptible to infection with *Leptospira bovis*, while Morton[2] has reported on the susceptibility of the Syrian hamster to infection with *Lept. icterohemorrhagiae* and *Lept. canicola*. Larsen[3] found that white mice were susceptible to *Lept. icterohemorrhagiae*, but reported that this susceptibility falls off quite rapidly as the age of the animal increases beyond three weeks. Stavitsky[4] found that not all strains of white mice were equally susceptible to fatal infections. Chick embryos and baby chicks also have been used.[5,6] The majority of these experiments have been carried out using diluted cultures of the organism, and no comparison has been made to determine which would be the most suitable animal. Probably the animal of choice will depend upon the leptospiral serotype, but this may not always be known. The following experiments were made in an attempt to compare several different laboratory animals as to their susceptibility to infection with *Lept. pomona*.

EXPERIMENTAL METHODS

Only one strain of *Lept. pomona*, obtained from the Rocky Mountain Laboratory, Hamilton, Montana, was used throughout these experiments. This organism has been maintained on artificial medium[7] for several years and was selected because its virulence should be stabilized. Most investigators state that the virulence of pathogenic leptospirae is reduced when these organisms are subcultured in artificial medium.

The inoculum for the laboratory animals consisted of known numbers of *Lept. pomona* obtained in the following manner: aliquots of 2- to 3-week-old cultures, incubated at room temperature, were killed with formalin to prevent motility; the leptospirae were counted using the Petroff-Hausser[8] bacterial counting chamber and darkfield illumination; the numbers per ml of culture was determined and the living culture so diluted as to have a known number of leptospirae per inoculum.

The laboratory animals used were 21 days of age or younger, and included guinea pigs, Syrian

Received for publication March 20, 1956.

Scientific Paper no. 1477, Washington Agricultural Experiment Stations, Pullman. Project no. 1199. This investigation was supported in part by funds provided for biological and medical research by the State of Washington, Initiative Messure no. 171.

1. Borg-Peterson, C. 1950, Pathogenicity of *Leptospira bovis* for guinea pigs and mice. Acta. path. et microbiol. Scandinav. **27**: 726–734.
2. Morton, H. E. 1942, Susceptibility of Syrian hamsters to leptospirosis. Proc. Soc. Exper. Biol. & Med. **49**: 566–568.
3. Larsen, C. 1941, Susceptibility of young mice (Mus musculus) to *Leptospira icterohemorrhagiae*. Pub. Health Rep. **56**: 1546–1556.
4. Stavitsky, A. B. and Green, R. G. 1945, Susceptibility of young white mice (Mus musculus) to experimental leptospirosis. Science, **102**: 352–353.
5. Hoag, W. G., Gochenour, W. S. and Yager, R. H. 1953, Use of baby chicks for isolation of leptospires. Proc. Soc. Exper. Biol. & Med. **83**: 712–713.
6. Gleiser, C. A., Jahnes, W. and Byrne, R. 1955, Avian leptospirosis: studies on chicken embryo culture. Cornell Vet. **45**: 296–303.
7. Ringen, L. and Gillespie, R. 1953, A simple medium for the cultivation of Leptospira. J. Bact. **67**: 252.
8. Simmons, J. S. and Gentzkow, C. J. 1944, Laboratory methods of U. S. Army. U.S. Govt. Print. Office: 404.

TABLE 1.—*A comparison of susceptibility of several different laboratory animals to infection with* Lept. pomona.*

Number of leptospirae†	Guinea pig	percent +	Hamster	percent +	White mice	percent +	Chicks	percent +
14,000	40/40‡	100§	46/46	100	72/73	99	0/5	0
7,000	38/38	100	35/36	97	58/59	98	0/10	0
3,500	36/37	97	24/27	89	48/51	94	0/15	0
1,750	30/32	94	15/23	65	35/42	83	0/20	0
875	23/27	85	8/27	30	24/34	71	0/25	0
550	17/24	71	3/26	12	21/33	64		
275	12/25	48	2/31	7	12/29	41		
100	3/20	15	2/37	5	7/27	26		

* Based on an accumulated series using 67 guinea pigs, 81 hamsters, 92 white mice, and 25 chicks.
† Number of *Lept. pomona* per inoculum.
‡ Numerator = number of animals infected; denominator = animals inoculated.
§ Percent of animals infected based on the demonstration of a rising blood serum antibody titre using the agglutination-lysis test.

hamsters, white mice (Webster Swiss), and 2-day-old White Leghorn chicks. A total of 67 guinea pigs , 93 hamsters, 92 mice, and 25 chicks were used, these being divided into groups of 5 to 10 animals per group. The inoculum was given intraperitoneally and infection was based upon the demonstration of a rising blood serum titer using the agglutination-lysis test.[9] The animals were bled at weekly intervals ranging from 2 to 6 weeks, and several serologically positive animals from each group sacrificed for cultural studies.

A comparison of susceptibility to *Lept. pomona* by these various animals was made by determining the infective dose$_{50}$ (ID_{50}), this being based on an accumulated series.[10]

RESULTS AND DISCUSSION

The detection of pathogenic leptospirae in contaminated materials can be accomplished through the use of laboratory animals, either by isolation of the spirochete from blood and tissues, or demonstrating a rising blood serum antibody titer in the inoculated animal. When death of the animal occurs, isolation of the spirochete is the most satisfactory method. However, *Lept. pomona* may not produce a fatal infection in an inoculated animal, and one of the most rapid methods for its detection is by demonstrating a rising titer. Guinea pigs and white mice become serologically positive after an average of 2 weeks post-inoculation, while hamsters require 3 to 4 weeks. Relatively large numbers of this organism were required to infect the chicks, and no detectable serological response was observed under the conditions of these experiments.

When inoculating large numbers of leptospirae, any of the above animals may be satisfactory. However, if the organisms are present in small numbers the guinea pig is probably the most suitable animal. Although no significant difference could be found between the ID_{50} of guinea pigs and white mice ($X^2 - 1.4165$),[11] a much larger inoculum can be given to the former, i.e., when trying to detect *Lept. pomona* in urine from chronic cases of bovine leptospirosis, 5.0 ml of urine can be inoculated into a guinea pig, as compared with 0.5 ml into white mice or hamsters. A highly significant difference was found between the ID_{50} of hamsters and guinea pigs ($X^2 - 10.6288$)[11] and between hamsters and white mice ($X^2 - 8.2077$),[11] the hamsters being more resistant (table 1). The ID_{50} was determined for these animals with the following results: guinea pigs, approximately 300 leptospirae; white mice, approximately 400 leptospirae; and hamsters, approxi-

9. Rocky Mountain Laboratory; Leptospira unit: Leptospirological Techniques. Circ. Letter, 1952.
10. River, T. M. 1948, Viral and Rickettsial Infections of Man. Philadelphia, J. B. Lippincott Co., p. 69.
11. Snedecor, G. W. 1950, Statistical Methods, Iowa State College Press, p. 190.

mately 1400 leptospirae, per inoculum. This method for determing the infective dose was tested experimentally in guinea pigs and white mice. Approximately 350 leptospirae (*Lept. pomona*) per inoculum were inoculated into each of 6 guinea pigs; four became infected. Approximately 1100 and 520 per inoculum were inoculated into each of 8 and 6 mice, respectively; 7 in the former, and 4 in the latter became infected.

The choice of a laboratory animal will depend upon several different factors, including availability, numbers of animals to be used, and type of experimental work. Where the numbers of leptospirae are sufficient to permit the use of a relatively small inoculum, white mice and hamsters both provide a satisfactory animal. However, for the isolation of *Lept. pomona*, the hamster probably is the animal of choice since these spirochetes seem to persist in the tissues of these animals for much longer periods of time. The isolation of *Lept. pomona* from guinea pigs and white mice is best made during the first 2 weeks postinoculation.

Since only one strain of *Lept. pomona* was used throughout these experiments, it is not known if other strains would give the same results.

SUMMARY

Guinea pigs, Syrian hamsters, white mice (Webster Swiss), and 2-day-old chicks were compared as to their susceptibility to infection with known numbers of *Leptospira pomona*. Infection in these animals was based upon the demonstration of a rising blood serum antibody titer, using the agglutination-lysis test. Under the conditions of these experiments, it was found that the guinea pig and white mouse are about equally susceptible, the hamster significantly more resistant, and the chicks highly resistant. When working with relatively large numbers of this organism, any of the above animals may be satisfactory.

THE ASSOCIATION IN YOUNG MICE OF INTESTINAL INFLAMMATION AND THE LOSS OF ADULT WORMS FOLLOWING AN INITIAL INFECTION WITH *TRICHINELLA SPIRALIS*

JOHN E. LARSH, JR., GEORGE J. RACE, AND WILLIAM B. JEFFRIES*

From the Department of Parasitology, School of Public Health, University of North Carolina, Chapel Hill, North Carolina, and the Department of Pathology, The University of Texas Southwestern Medical School, Dallas, Texas

As far as known, there are no reports of studies comparing directly the longevity of adult *Trichinella spiralis* in the intestinal tract of young and old mice. However, it is known: (1) that there is no significant difference in the adult worm burdens of young and old mice 5 days after an initial infection (Larsh and Hendricks, 1949), (2) that in old mice there is a sudden and significant loss of adult worms between 11 and 14 days after infection (Larsh et al, 1952), and (3) that at 15 days following infection young mice harbor more worms than old mice (Riedel, 1950). At first thought, the latter observation may suggest the operation of age immunity in the old mice as demonstrated in several host-parasite combinations. However, recent studies in old mice have revealed evidence that the immune phenomena have begun to develop during the course of the initial infection (Larsh and Race, 1954). Circulating antibodies in low titer were detected, and an inflammatory response was observed, which differed only in the time of its appearance and its severity from that observed in strongly immunized mice. It is important to add that in both the immunized and nonimmunized mice the peak of the inflammatory response occurred shortly before it is known that the worms are lost from the same area of the intestine. The fact that the loss of worms from an initial infection can be prevented by injecting cortisone to suppress the inflammatory response (Coker, 1955) suggests strongly that in old mice the actual expulsion of the worms is due mainly to the cellular reaction associated with the inflammation.

In view of the observations in old mice, it would seem permissible to speculate that the slower rate of elimination of worms from young mice given an initial infection is related to a delayed development of acquired immunity. It is generally agreed that young animals respond more slowly to antigenic stimulation than their mature counterparts, and in at least one host-parasite combination this relationship is clear (Cort and Otto, 1940). Nevertheless, conclusive proof is lacking in the present case, since no evidence has been presented to show that acquired immunity is operating at the time the adult worms are eliminated from young mice. The present experiment was designed, therefore, in an attempt to determine whether or not this is the case. It appears that a striking cellular reaction in the intestinal tissue occurs only in the presence of acquired immunity (Larsh and Race, 1954) and that such a reaction is necessary to cause the expulsion of adult worms (Coker, 1956). Therefore, in the present study it was decided to use as the criterion of acquired immunity the presence of a characteristic cellular response in the intestinal tissue.

Received for publication March 26, 1956.

* Present address: Zoology Department, Indiana University, Bloomington, Indiana.

MATERIALS AND METHODS

The various experimental procedures were those described in earlier reports from this labo-

ratory (Larsh and Kent, 1949; and others). Student's *t* test was used to determine the statistical significance of the observed differences in numbers of parasites recovered from the mice at the various periods after infection. The results from each necropsy period were compared with those for the succeeding period. A probability greater than 0.05 was not considered significant.

The intestinal tissue for study was prepared as follows. The proximal second one-fourth of the small intestine was removed from each animal. It was left unopened and cut into three small (3mm) pieces. These were fixed in Helly's fluid (formalin, potassium bichromate, and bichloride of mercury) for 15 hours, after which the tissue was washed in running tap water for 24 hours. The tissue was passed slowly through an ethyl alcohol series (25, 50, 80, 95, and 100%, 1 to 3 hours in each concentration) into 100% alcohol and xylene (1 hour) and finally through xylene (1½ hours) into tissuemat (58 C) for embedding. Histologic cross section preparations 6μ thick were made, which were stained with hematoxylin and eosin.

EXPERIMENTAL PROCEDURES AND RESULTS

Thirty-six mice, 5 weeks old and of approximate weight, were each given by mouth an infection of 300 *T. spiralis* larvae. On both the 3rd and 5th days following infection, two mice (one of each sex) were sacrificed and the intestinal tissue was removed for histopathologic study. Beginning 7 days after infection and continuing through the 21st day, four mice (two of each sex) were sacrificed every other day, i.e., on days 7, 9, 11, 13, 15, 17, 19, and 21. At each period, two mice were used to determine the number of adult *T. spiralis* present in the small intestine and two were used to obtain tissue for histopathologic study.

At 7, 9, 11, 13, 15, 17, 19, and 21 days after infection, the following average numbers of adult worms were recovered from the small intestines of the two mice (the percentages of development are given in parentheses following the average counts): 269.5 (89.8); 229.0 (76.3); 259.5 (86.5); 238.5 (79.5); 232.5 (77.5); 81.5 (27.2); 59.0 (19.7); and 83.5 (27.8). The only comparison showing statistical significance was that between the counts at 15 days (232.5) and 17 days (81.5). This was found to be significant at the 1% level. It is clear, therefore, that in these young mice there was a sudden and significant loss of worms between 15 and 17 days after initial infection.

A detailed study was made of the intestinal tissue prepared from all 20 mice used in this portion of the experiment. A brief description of the histopathologic findings is given below. On the basis of these observations, representative photomicrographs were prepared (figures 1 through 6).

Three days after infection (*fig. 1*).—There were moderate numbers of parasites in the lumen of the intestine and a few were invading the mucosa. The mucosa, with very few polymorphonuclear leukocytes, showed little cellular reaction. An occasional plasma cell was seen. Lymphoid tissue, present normally in this region of the intestine, was also observed. Therefore, the small number of lymphocytes noted did not indicate inflammation.

Five days after infection (*fig. 2*).—Small numbers of polymorphonuclear leukocytes were seen in localized areas. Plasma cells and lymphocytes were observed, but their small numbers would indicate that they were normal to the intestine and not the result of inflammation. An occasional monocyte was also noted in these slides. Edema of the mucosa appeared to be developing, as indicated by a thickening of the mucosa with loose tissue spaces especially in the papillary folds.

Seven days after infection (*fig. 3*).—Polymorphonuclear leukocytes were beginning to occur in small to moderate numbers, indicating the initiation of mild inflammation. Despite this, in

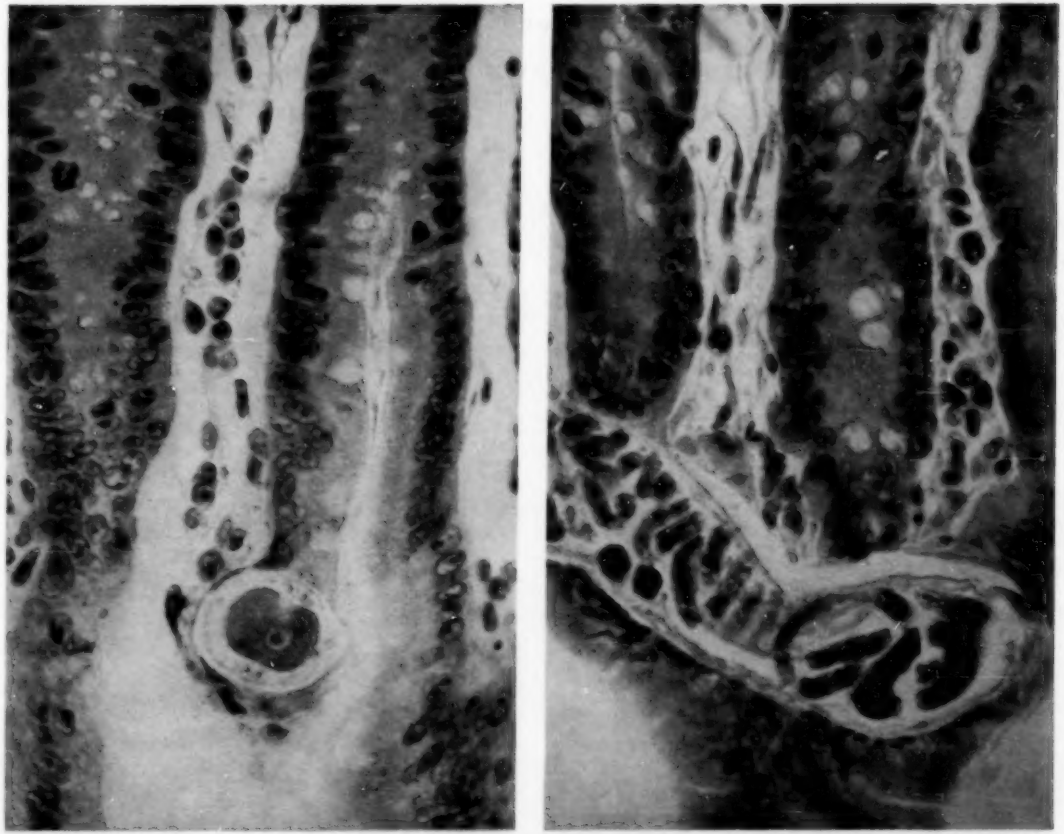
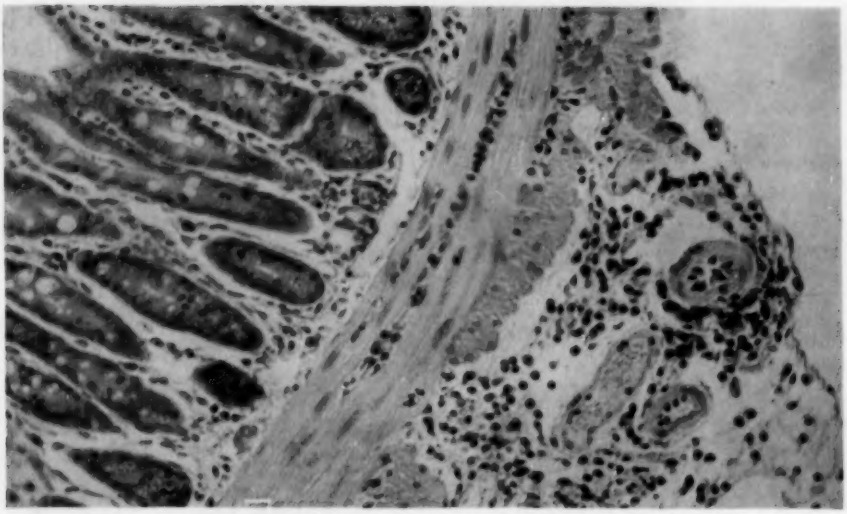


FIG. 1.—Intestinal mucosa 3 days after infection. Note the parasite in the mucosa and the relative absence of inflammation. ×660.

FIG. 2.—Five days after infection. Despite the proximity of the worm, there is only an occasional inflammatory cell present. ×660.



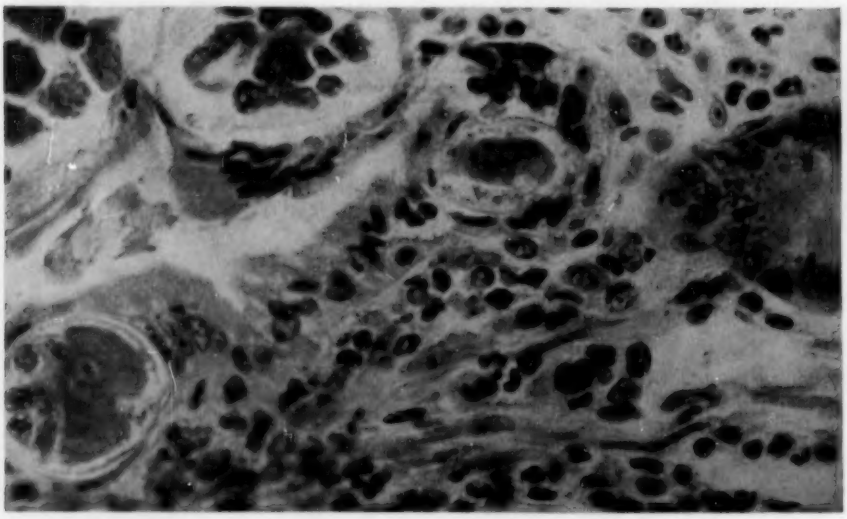


FIG. 3.—Seven days after infection. Note the cross-sections of worms and the early polymorphonuclear leukocytic exudate adjacent to them. ×660.

FIG. 4.—Eleven days after infection. The entire mucosa and the subserosal fat are infiltrated with leukocytes of mixed type. ×270.

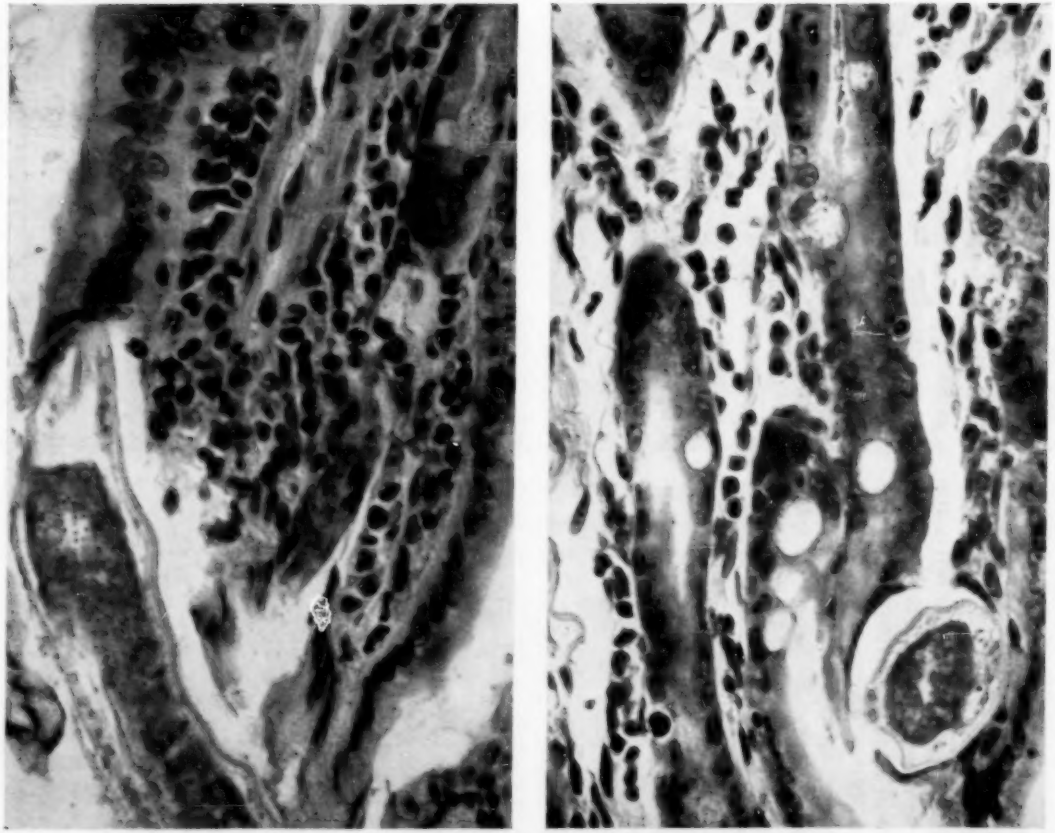


FIG. 5.—Eleven days after infection, higher power. In addition to the polymorphonuclear leukocytes, a few plasma cells and histiocytes are present. ×660.

FIG. 6.—Seventeen days after infection. Note that the inflammatory reaction is less intense despite the persistence of some worms within the mucosa. ×660.

some areas the parasite was seen invading the mucosa without obvious inflammation immediately adjacent to it. The monocytes or macrophages, the plasma cells, and the lymphocytes still showed little change from the normal.

Nine days after infection.—By this time, the polymorphonuclear leukocytic response was diffuse and moderate to marked in all areas, including the submucosa. The cellular reaction was acute and becoming generalized to involve all of the intestine. Parasites were noted both in the mucosa and in the lumen. The mucosa was beginning to show an increase in the number of mononuclear cells of the monocyte type, and a few plasma cells were noted.

Eleven days after infection (*figs. 4 and 5*).—At this stage, the polymorphonuclear leukocytic response was diffuse and marked. There would seem to be no doubt that this stage represents the zenith of the cellular reaction. There was a definite acute inflammatory reaction in the submucosa as well as in the mucosa. Also noted at this stage was an increase in the monocytic macrophage type of cells and lymphocytes. In one slide, in which parasites were deep within the mucosa, the mesenteric fat was inflamed markedly and polymorphonuclear leukocytes and lymphocytes were seen surrounding small blood vessels.

Thirteen days after infection.—There was a diminution in the number of polymorphonuclear leukocytes as compared with the numbers observed from the 7th through the 11th day, and there was an increase in the lymphocytes and monocytes. The mucosa was compact and not as loose and edematous as in some of the previous stages.

Fifteen days after infection.—The polymorphonuclear leukocytic response had decreased definitely by this time, but it was still noted in some focal areas. A mixture of lymphocytes, monocytes, and an occasional plasma cell was beginning to occur, and the lymphoid tissue seemed to be more active than in the previous stages. Furthermore, the submucosa had a mixture of lymphocytes and monocytes.

Seventeen days after infection (*fig. 6*).—The polymorphonuclear leukocytes had continued to diminish in numbers, but the other cells seemed little changed from 15 days.

Nineteen and twenty-one days after infection.—The polymorphonuclear leukocytes, while still present, had continued to decrease in numbers. The inflammatory reaction was of a subacute diminishing type with a mixture of mostly plasma cells and monocytes.

In summary, the inflammatory reaction in the intestinal tissue of these young mice developed slowly. It was first recognized 7 days after initial infection and gradually reached the acute phase, which at 11 days was at the peak level. The predominant cell during the acute phase was the polymorphonuclear leukocyte. From the 11th day to the end of the observations on the twenty-first day after infection the acute phase changed gradually into a subacute inflammatory reaction, which diminished considerably in intensity toward the end of the period of observation. The cells of the subacute phase were of mixed type, especially lymphocytes, plasma cells and monocytes.

A few additional observations are worthy of mention. The appearance of the developing worms through the eleventh day was similar to that noted in old mice given an initial infection (Larsh and Race, 1954). In brief, the worms seemed to be much larger than those of the same age observed earlier in immunized mice, and from the appearance of the large number of eggs and embryos there was no apparent

interference with the normal reproductive potential of the females. However, after the 11th day (i.e., 13 to 21 days) the worms in the mice of the present study appeared to show evidence of being inhibited, but the small numbers observed on days 17, 19, and 21 did not permit a definite conclusion. Mechanical tissue damage was noted as in the previous study of nonimmunized old mice, but a direct comparison can not be made due to a difference (300 versus 400) in the dose of larvae used for infecting the two groups. However, it is clear that the cellular reaction was much less striking in the young mice, and it is unlikely that the difference in the number of larvae used for infection could have accounted for such a difference in the tissue response. Finally, as in the nonimmunized mice of the previous study, the number of inflammatory cells was no greater in the areas adjacent to the worms than in the mucosa in general, eosinophils were not abundant in any of the sections, and material resembling protein precipitate was not observed in the tissue.

DISCUSSION

The young mice of the present study showed a significant loss of adult worms from the small intestine between 15 and 17 days after initial infection with 300 *T. spiralis* larvae. In old mice of the same strain this loss occurs between 11 and 14 days after initial infection (Larsh et al, 1952). It is interesting to compare the percentages of development and longevity of the adult worms in these two groups of mice. There is no significant difference in the counts through the 11th day, and after the significant loss of worms had occurred in both groups the counts again are similar. Thus, the only difference is the time required to eliminate a significant number of the worms that established, this being about three days earlier in the old mice. It is clear, therefore, that if a comparison in worm burdens is made shortly after the old mice lose most of their worms and before the worms are lost from the young mice a striking difference can be demonstrated, which by itself may be misleading. This was the case in the study by Riedel (1950), in which counts were made only at 15 days after infection. In the absence of other criteria, there is no justification for relating such a difference in counts to the operation of age immunity. As a type of natural immunity an effect(s) due to age alone would be expected to manifest itself soon after establishment of the worms and not be delayed for more than eleven days. On the other hand, such a delay would be expected in the presence of acquired immunity, since it has a characteristic slow development.

The histopathologic observations of the young mice of the present study were similar to those made in old mice given an initial infection with *T. spiralis* (Larsh and Race, 1954). In both cases, there was a mild early reaction, a moderately severe, acute reaction, and a subacute or chronic diminishing reaction. Furthermore, in both cases the cellular components of the three stages were similar; the polymorphonuclear leukocyte was the prominent cell during the acute phase, and mixed mononuclear cells characterized the subacute or chronic phase. Finally, the appearance of the worms, the degree of mechanical tissue damage, and other pertinent observations mentioned above were similar in both groups of mice. Therefore, it is clear that the only difference in the inflammatory response of the two groups was one of time and severity. In the old mice, mild inflammation was initiated between 4 and 6 days after infection and reached a zenith at 8

days, while in the young mice mild inflammation developed between 5 and 7 days after infection and the cellular reaction did not reach its peak until 11 days. It is important to recall that not only was the cellular response delayed in the young mice, but at its peak it failed to reach the severe proportions noted in the old mice. In both groups of mice, the peak of the cellular reaction occurred about 5 days prior to the time that there is a sudden and significant loss of worms from the same region of the intestine. It is clear, therefore, that: (1) the worms from an initial infection are eliminated only after the tissue remains in a highly inflamed condition for several days, and (2) the more rapid loss of worms from old mice is related to a more rapid development of this inflamed condition. The fact that cortisone, a hormone that exerts a powerful suppressive action upon the lymphoid-macrophage system, prevents the characteristic loss of worms from an initial infection (Coker, 1955) suggests strongly that the cellular reaction brings about the actual expulsion of the worms. That such a reaction is a manifestation of acquired immunity is evident from the following account of its role in the immunity demonstrated by previously immunized mice.

The cellular reaction in the intestinal tissue of previously immunized mice has been observed soon after reinfection and it reaches a peak at 4 days (Larsh and Race, 1954). At this time the reaction, while similar in nature, is considerably more severe than that noted at the peak level (8 days) in mice of the same age given only one infection. About 6 days after reinfection, or 2 days after the peak of the inflammation is reached, strongly immunized mice lose their worms in significant numbers from the same region of the intestine (Larsh et al, 1952). If such strongly immunized mice are given injections of cortisone the characteristic cellular reaction is suppressed almost completely and the worms persist in large numbers for at least 14 days after reinfection (Coker, 1956). Since no effect of cortisone upon the humoral factor(s) was demonstrated, there would appear to be little doubt that there is a cause and effect relationship between the characteristic cellular reaction and the loss of worms. In any event, there is no question that this reaction is an important manifestation of acquired immunity.

It is clear from the above that the relationship of a characteristic cellular response in the intestinal tissue and the loss of adult worms is evident in old immunized mice, in old mice given one infection, and in the young mice of the present study given one infection. The evidence indicates further that the main difference between these three groups is in the time of appearance and severity of the cellular response, which in turn influences directly the persistency of the worms in the same region of the intestine. Therefore, there would seem to be no doubt that this difference is due to the degree of acquired immunity present in the three groups. For this reason, it is safe to assume that following initial infection the more rapid elimination of worms by old mice is not due to factors associated with age immunity but to the ability of the old mice to develop acquired immunity more rapidly than young mice. This is to be expected if one accepts the view expressed by many workers that young animals do not respond to antigenic stimulation as effectively as their mature counterparts.

SUMMARY

Young mice given an initial infection with *Trichinella spiralis* lost a significant number of adult worms between 15 and 17 days after infection. Related

to this loss of worms was the development in the intestinal tissue of a characteristic cellular reaction, which reached its peak at 11 days after infection. Evidence is presented to show that this reaction, differing only in the time of its initiation and in degree from that in old mice given one infection and in those strongly immunized by prior infections, is a manifestation of acquired immunity. Therefore, these results indicate that following initial infection of young and old mice the slower elimination of adult *T. spiralis* from the young mice is due to their inability to develop acquired immunity as rapidly as the old mice.

REFERENCES

Coker, C. M. 1955, Effects of cortisone on *Trichinella spiralis* infections in non-immunized mice. J. Parasitol. **41**: 498–504.

Coker, C. M. 1956, Some effects of cortisone in mice with acquired immunity to *Trichinella spiralis*. J. Infect. Dis. **98**: 39–44.

Cort, W. W. and Otto, G. F. 1940, Immunity in hookworm disease. Rev. Gastroenterology, **7**: 2–13.

Larsh, J. E., Jr. and Hendricks, J. R. 1949, The probable explanation for the difference in the localization of adult *Trichinella spiralis* in young and old mice. J. Parasitol. **35**: 101–106.

Larsh, J. E., Jr. and Kent, D. E. 1949, The effect of alcohol on natural and acquired immunity of mice to infection with *Trichinella spiralis*. J. Parasitol. **35**: 45–53.

Larsh, J. E., Jr., Gilchrist, H. B. and Greenberg, B. G. 1952, A study of the longevity of adult *Trichinella spiralis* in immunized and non-immunized mice. J. Elisha Mitchell Sc. Soc. **68**: 1–11.

Larsh, J. E., Jr. and Race, G. J. 1954, A histopathologic study of the anterior small intestine of immunized and non-immunized mice infected with *Trichinella spiralis*. J. Infect. Dis. **94**: 262–272.

Riedel, B. B. 1950, Further studies of the effect of age of mice upon adult *Trichinella spiralis*. J. Parasitol. **36**: 27–28.

STUDIES ON *PASTEURELLA PESTIS* IN VARIOUS FLEA SPECIES

III. TRANSMISSION OF AVIRULENT STRAINS OF *PAST. PESTIS* BY *XENOPSYLLA CHEOPIS*

SUYEMOTO, W.,* C. M. WHEELER,† D.C. CAVANAUGH,‡ YOKO YAMAKAWA,‖ AND TOMIKO SHIMADA‖

From the Departments of Entomology and Bacteriology, 406th Medical General Laboratory, APO 343, San Francisco, California

Experimental transmission of virulent plague organisms by various flea species has been reported by many workers. A careful search of the available literature failed to disclose any information concerning the transmission of avirulent plague organisms by fleas to experimental animals.

Quan, Kartman and McManus (1954) observed that proventricular blockage of *Xenopsylla cheopis* occurred with avirulent plague strain A1122, but they reported no transmissions. Cavanaugh et al (1956) confirmed these findings.

The successful transmission of the avirulent *Pasteurella pestis* strain A1122 to experimental animals is reported here.

MATERIALS AND METHODS

A simplified artificial feeding apparatus designed by Wheeler et al (1956) was made. Fleas were fed a suspension of agar-grown *Past. pestis* in defibrinated rabbit blood through a freshly prepared albino mouse skin membrane. After a feeding period of 24 hours, engorged fleas were removed, placed in test tubes with a small quantity of fine wood shavings and kept at 21 to 24 C for 3 to 6 days. Fleas were kept in a desiccator jar containing 100 ml of a 5% KOH solution to provide a relative humidity of approximately 65% at 25 C.

Past. pestis strain A1122 is an avirulent variant, noted for its immunogenic properties. This strain was isolated by Dr. Karl F. Meyer, University of California. Other than virulence, this strain possesses every known characteristic of the virulent organism and is especially rich in envelope antigen. The strain used in these experiments was a lyophilized culture originally acquired from Dr. Meyer and maintained in this laboratory.

The fleas used in this study were *X. cheopis* of two strains colonized in this laboratory; one strain was from San Francisco, California, and the other from Kobe, Japan. Tests for the presence of *Past. pestis* in the fleas were performed by dissection of the proventriculus and stomachs. These organs were washed in three changes of saline solution and macerated in 1% peptone water. The ground tissue was then inoculated into tryptose phosphate broth (Difco). Complete bacteriological identification tests, including biochemical reactions and the bacteriophage test of Gunnison et al (1951), were performed on each organism isolated. Similar bacteriological procedures were carried out on organisms isolated at autopsy of the mice.

Virulence of the organisms isolated was determined by the inoculation of 0.2 ml of a 24 hour 37 C broth culture in tryptose phosphate broth. Doses were given by both the intraperitoneal and subcutaneous routes, four mice being inoculated by each route. Mice were observed for 21 days, then sacrificed and autopsied.

In order to allow the fleas to feed on each mouse and to prevent the mouse from eating the fleas, a confinement cage, 15 cm×3 cm×3 cm was made of reinforced hardware cloth.

PROCEDURE

Experimental feedings were conducted with *X. cheopis* (California strain) and with *X. cheopis* (Kobe, Japan strain) to determine the rate of infection, blocking and survival of fleas infected through artificial membranes. Following the infectious meal, fleas were divided into

Received for publication April 9, 1956.

* Entomologist, 406th Medical General Laboratory.

† Advisor in Medical Entomology, 406th Medical General Laboratory.

‡ 1st Lt., MSC, USA, Bacteriologist, 406th Medical General Laboratory.

‖ Technicians, 406th Medical General Laboratory.

three groups. Group 1 (62 fleas) was maintained under optimum conditions in test tubes for 96 hours. Groups 2 and 3 were maintained on mice for 120 hours; group 2 (28 fleas) was allowed to feed ad libitum, while group 3 fleas (48) were fed individually once daily. Infection was determined by culture of flea stomachs. Gastrointestinal blockage was determined, 3 to 6 days after feeding, by microscopic examination of fleas, many of which had been observed to make repeated and, occasionally, frantic attempts to feed. Such fleas were usually found to have no fresh blood in their stomachs or a relatively small amount when compared with non-infected fleas similarly fed. On dissection, many of these fleas contained huge masses and clots in the stomach which appeared to consist principally of bacilli. Following these determinations, transmission studies were made using local Japanese strains of albino mice (Strains ddN and SM).

Male and female fleas in lots of 25 to 70 were starved for 3 or 4 days prior to introduction into the artificial feeding apparatus. Following a 24-hour feeding period, engorged fleas were held for 3 to 6 days and microscopic examination of all engorged fleas was made to detect development of proventricular blockage. Cultural confirmation for presence of *Past. pestis* was carried out on a representative sample of apparently blocked fleas from each lot. In subsequent transmission attempts with presumably "blocked" fleas, fleas from original infected lots were pooled in lots of 33 to 113 and each lot allowed to feed en masse on single mice confined in special cages. Upon death of the mouse or upon sacrifice, a new mouse was placed in the confinement cage. Each removed mouse was examined for the presence of *Past. pestis*. At 4, 6, 21 and 46 days, 4 to 5 fleas (depending upon the total fleas surviving) were examined for blocking and dissected for culture.

This procedure was carried out once with *X. cheopis* (Japan strain) and three times with the *X. cheopis* strain from California. In the transmission experiment with *X. cheopis* (Japan strain) the concentration of viable plague organisms in the feeding apparatus was 725×10^7 per ml of blood. The fleas were held for 3 days after the infectious meal before exposure to the first mouse and 88 infected fleas were used. In the three transmission experiments with *X. cheopis* from California the feeding concentration of *Past. pestis* ranged from 850×10^7 to 109×10^8 per ml. In two experiments these fleas were held 3 days prior to exposure to the mice and in the third experiment the fleas were held 6 days.

RESULTS

Table 1 presents the findings of infection and blocking of fleas. Thirty-seven percent of the fleas of group 1 acquired the infection, but determination of blockage was not attempted. In group 2, 17.8% of the fleas were blocked; while in group 3, 14.6% developed gastro-intestinal blocks. Blockage was first observed to occur as early as the

TABLE 1.—*Data on* X. cheopis *found harboring* Past. pestis *after an infectious meal through an artificial membrane.*

	Group 1	Group 2	Group 3
	Fleas found infected 96 hours after meal	Fleas feeding *ad lib.* on mouse 120 hours after meal*	Fleas individually fed on mouse once daily 120 hours after meal*
Number tested per group	62	28	48
Number considered blocked by appearance and with positive culture	23	5	7
Percent positive	37	17.8	14.6
Percent blocked	†	17.8	14.6

* Time of dissection and culture.
† Not attempted.

TABLE 2.—*Data on incidence of* Past. pestis *in infective fleas during transmission experiments.*

Exp.	Day following infective meal			
	4	6	21	46 (termination)
1	3/4*	4/4	0/4	0/4
2	0/4	0/4	†	†
3	0/4	0/4	0/4	5/6
4	0/4	0/4	0/4	0/4

* Positive/tested.
† Not attempted, fleas all dead.

3rd day and as late as the 9th day following the infectious blood meal, while death of blocked fleas usually occurred about the 20th day.

Table 2 presents the data concerning the incidence of *Past. pestis* in the flea samples removed from groups of fleas in the transmission experiments. The samples were of necessity kept small so that the flea population would not be critically reduced. The small samples showed great variation in infection rates of the fleas. In some instances, such as early in series 1 and late in series 3, an unusually high ratio of infected fleas was found.

Single mice in the series exposed to the infected *X. cheopis* were found to harbor avirulent *Past. pestis* in their spleens during the period from the 12th to the 24th days of exposure. As seen from the data presented in table 3, no mice became positive before 12 days nor after 24 days following exposure to infected fleas. No mice presented the appearance of death by acute plague. No organisms were recovered from heart blood, peritoneal or pleural cavities. It is felt that the virulence of strain A1122 was not enhanced. No deaths were observed in mice challenged with heavy suspensions of the recovered strains.

DISCUSSION

Blockage of fleas with avirulent plague bacilli occurs in the approximate time required for blockage of fleas infected with virulent strains. It is not believed, however, that the ratio of blocked fleas is as great as occurs with virulent bacilli (Wheeler and Douglas, 1945). Although exposed to massive numbers of bacilli, some fleas appear to clear themselves with relative ease. Definite blockage did occur, however, at the rate of 14.6 to 17.8%.

The number of organisms administered by fleas to each mouse in the experiments is unknown, as is the biting incidence. The absence of lesions in exposed mice and the avirulence of recovered organisms indicate that no enhancement of virulence occurred.

No attempt was made to determine the mechanism of transfer of organisms from the fleas to the mice. It is not thought that the mice became infected by eating infected fleas because the restraint of the mouse limited its movement.

TABLE 3.—*Infected fleas and time interval involved in transmissions of avirulent* Past. pestis.

Flea strain	Concentration per ml blood of viable plague organisms used to infect fleas	Holding period in days of fleas after infectious meal at 21–24 C	Number of infected fleas used	Longevity in days host mice after exposure to fleas	Host mice in transmission series: died or sacrificed	Transmission
1				4	1. Died	Negative
X. cheopis Japan	725×10	3	88	8	2. Died	Negative
				5	3. Sacrificed	Positive
				25	4. Sacrificed	Negative
2				24	1. Sacrificed	Positive
X. cheopis California	850×10	3	33	3	2. Died	Negative
				21	3. Died	Negative
3				3	1. Died	Negative
X. cheopis California	100×10	3	113	21	2. Died	Positive
				8	3. Sacrificed	Negative
4				12	1. Died	Negative
X. cheopis California	109×10	6	61	5	2. Sacrificed	Positive
				24	3. Sacrificed	Negative

SUMMARY AND CONCLUSIONS

Avirulent *Pasteurella pestis* strain A1122 can cause gastrointestinal blockage in fleas and can be transmitted to mice by *Xenopsylla cheopis*. Fleas were experimentally infected en masse in an artificial feeding apparatus. No attempt was made to determine the mode of transmission. No enhancement of virulence was noted in A1122 by flea passage.

REFERENCES

Quan, S. F., Kartman, L and McManus, A. G. 1954, Studies on *Pasteurella pestis* in fleas. II. Experimental blocking of *Xenopsylla cheopis* with an avirulent strain of *Pasteurella pestis*. Science, 120(3131): 1101–1102.

Cavanaugh, D. C., Wheeler, C. M., Suyemoto, W., Shimada, T. and Yamakawa, Y. 1956, Studies of *Pasteurella pestis* in various flea species. I. Infection of flea species with avirulent strains of *Pasteurella pestis*. J. Infect. Dis. **98**: 103–106.

Wheeler, C. M., Suyemoto, W., Cavanaugh, D. C., Shimada, T. and Yamakawa, Y. 1956, Studies on *Pasteurella pestis* in various flea species. II. Simplified method for the experimental infection of fleas. J. Infect. Dis. **98**: 107–111.

Wheeler, C. M. and Douglas, J. R. 1945, Sylvatic plague studies. V. The determination of vector efficiency. J. Infect. Dis. **77**: 1–12.

THE SIGNIFICANCE OF ANTIGEN-ANTIBODY REACTIONS IN TUBERCULOSIS

FLORENCE B. SEIBERT

From the Henry Phipps Institute, University of Pennsylvania, Philadelphia, Pennsylvania

Potent antigens, such as the tuberculoprotein and tuberculopolysaccharide could conceivably be present in tuberculous tissues containing disintegrating tubercle bacilli. And if, perchance, antibody-producing cells, which are attracted to the site, release their antibodies, the simultaneous presence of antigen and antibody could be expected to give rise to a precipitate in situ, similar to that formed when the two are mixed in vitro. Because of the accompanying cellular reaction to such a precipitate, it would become fixed locally. Furthermore, when the bacillary population would increase sufficiently to permit the presence of an excess of either antigen, it is possible that resolution of the antigen-antibody complexes might occur, resulting in liquefaction of the precipitate, as occurs in vitro in the well-known "zone phenomenon." The conclusion that such an antigen-antibody precipitation in situ and the resolution of the precipitate by antigen excess may play a role in the phenomena of caseation and liquefaction in tuberculosis would depend upon the demonstration of immunological specificity of the reactions involved.

That the isolated tuberculoprotein is a highly potent antigen capable of eliciting high concentrations of antibodies[1] as well as of calling forth the cells recognized to be antibody producers,[2–5] and, moreover, of accelerating the development of caseation and hastening death in infected animals[6] has been demonstrated. In addition to the tuberculoprotein, recent studies[1] suggest that the tuberculopolysaccharide may also play an important role in the disease because of its capability of suppressing immunological reactions, such as the complement-fixation and precipitin reactions.

PLAN OF EXPERIMENTS

In order to test the hypothesis set forth above, concerning the importance of the presence of tuberculoprotein and polysaccharide antigens and antibodies naturally present in vivo in infected animals, experiments were conducted (a) to isolate from tuberculous caseous material and identify tuberculoprotein and tuberculopolysaccharide antigens, antibodies to these antigens, and anti-

Received for publication April 16, 1956.

Supported in part by a grant from the National Tuberculosis Association made possible by a special bequest from the estate of Sophie Goman, and in part by a research grant (no. E-680) from the Microbiological Institute of the National Institutes of Health, Public Health Service.

1. Seibert, F. B., Miller, E. E., Buseman, U., Seibert, M. V., Soto-Figueroa, E., and Fry, L. 1956. The significance of antibodies to tuberculoprotein and polysaccharide in resistance to tuberculosis. Interference with antibodies by these antigens. Am. Rev. Tuberc. **73**: 547–562.
2. Sabin, F. R. 1941, Cellular reactions to fractions from tubercle bacilli. Am. Rev. Tuberc. **44**: 415–23.
3. Miller, F. R. 1931, The induced development of plasma cells. J. Exper. Med. **54**: 333–47.
4. Leduc, E. H., Coons, A. H. and Connolly, J. M. 1955, II. The primary and secondary responses in the popliteal lymph node of the rabbit. J. Exper. Med. **102**: 61–71.
5. Ehrich, W. E. 1953, Cellular sources of antibodies in blood cells and plasma proteins. Edited by J. L. Tullis. Acad. Press. Inc. New York, p. 187.
6. Seibert, F. B. 1933, Effect of sensitization with tuberculin protein upon development and course of experimental tuberculosis. Proc. Soc. Exp. Biol. & Med. **30**: 1274–6.

gen-antibody complexes, (b) to study the solubilities in antigen excess or in high salt concentration of antigen-antibody complexes which can be formed in vitro, (c) to study the nature, especially the solubilities in excess antigen, of such complexes isolated from caseous lungs.

Animals.—Since a simultaneous experiment (reference 1 and unpublished results) was in progress in our laboratory to test the relationship of antibodies to the immune process in tuberculosis, the serums and tissues of these rabbits were available for the study reported in this paper. There were 10 untreated rabbits serving as controls, which were infected intravenously with a virulent strain (Ravenel) of bovine tubercle bacillus. Among these were two rabbits (no. 53 and 54) used in this study. Fifteen other rabbits were sensitized by 15 weekly intracutaneous injections of 0.25 mg live BCG organisms, two with 1 mg doses of heat-killed BCG organisms, and three (including no. 2 used in this study) with 17 intracutaneous injections of the A type of tuberculoprotein, before being infected at the same time as the controls. Among these 20 treated rabbits, 8 (including rabbits no. III6 = 51* and 49 used in this study) were given 13 subsequent weekly intracutaneous injections of 0.25 mg heat-killed BCG organisms; two (including rabbit no. 46 used in this study) were given a similar number of injections of 4 mg tuberculopolysaccharide; one (rabbit no. 41) received a similar number of injections of 0.5 mg tuberculoprotein; and nine rabbits (including no. 2, 17 and 31 used in this study) received no treatment following infection.

Since blood had been taken from these rabbits at regular intervals during their sensitization and infection periods, their frozen serums were available. It was possible, therefore, to select for this study serums known to contain antibodies in high titer only to tuberculoprotein (rabbits no. 2 and 41), or to both tuberculoprotein and tuberculopolysaccharide (rabbits no. 17, 31, 49 and III6 = 51).

Moreover, the organs of these rabbits were also available, since at death they were removed and frozen immediately. The lungs of rabbit no. 54, which was a control rabbit and had no treatment other than infection with tubercle bacilli, and of rabbit no. 41, which had been injected with BCG prior to and with tuberculoprotein after infection, were chosen for study.

The lungs of two guinea pigs with extensive tuberculosis which had no treatment prior to nor after infection with a virulent human strain (H37 Rv) of tubercle bacillus, were also pooled and extracted.

Two large sacs filled with caseous material, which were found in the lower abdominal region adhering to the mesentery of a control rabbit no. 53, were also selected for study. A small caseous nodule on the ribs of rabbit no. 46, which had been injected with BCG prior to, and with tuberculopolysaccharide after infection was also studied.

Materials and methods.—The antigens used were purified tuberculoproteins, 107 II (A type), 109 II (B type), PPD-S, and tuberculopolysaccharide (I) previously described.[1]

The presence of antibodies in serum or tissue extracts was detected by the direct precipitin test, using the antigens mentioned above.

The presence of antigens in tissue extracts was detected by the direct precipitin test, using the serums, described under "animals," which were known to have high antibody content.

* III6 = 51 is one of the rabbits given to us by Dr. Max B. Lurie from his series of resistant rabbits.[7]

7. Lurie, M. B., Zappasodi, P. and Tickner, C. 1955, On the nature of genetic resistance to tuberculosis in the light of the host-parasite relationships in natively resistant and susceptible rabbits. Am. Rev. Tuberc. **72**: 297–329.

TABLE 1.—*Precipitin tests.*

Tissue and animal	Extracts	With antiserum for antigens: Normal No. 84	Antiprotein: No. 2	Antiprotein: No. 41	Antiprotein and Antipolysaccharide: No. 17	Antiprotein and Antipolysaccharide: No. 31	Antiprotein and Antipolysaccharide: No. 111 6=51	Antiprotein and Antipolysaccharide: No. 49	Control on Extract	With antigens for antibodies: Tuberculoproteins: A (107 11): 50	A (107 11): 10	A (107 11): 5	A (107 11): 1	B (109 11): 50	B (109 11): 10	B (109 11): 5	B (109 11): 1	PPD-S: 50	PPD-S: 10	PPD-S: 5	PPD-S: 1	Tubercule polysaccharide 1: 50	Tubercule polysaccharide 1: 10	Tubercule polysaccharide 1: 5	Tubercule polysaccharide 1: 1
Lungs—guinea pigs	*a*		+							±				±	+							+	±		
	b	Crystals			−	+				−	−			−								−	−		
		Crystals	−		+	+	+			−	−			−								−	−		
Lungs—rabbit no. 41	1	−		+		+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	3	−		±	+	+	+			+	+	+	±	+	+	±		+	+	+	+	+	+	+	+
Lungs—rabbit no. 54	1	−		±						−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	3	−			+	+	+			−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Caseous material in sac—rabbit no. 53	1	−		+				+		±	±	±	+	±	±	±	+	±	±	±	+	±	±	±	+
	3	−		+	+	±		+		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Control on serum and antigens		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

The Klett colorimeter was used for determining the turbidity of the suspensions with filter λ660.

EXPERIMENTAL

Identification of antigens and antibodies in tuberculous lungs of guinea pigs.—The extensively involved lungs from two tuberculous guinea pigs were pooled and homogenized in phosphate buffer. The extract *a* was filtered through the Seitz filter, centrifuged in the Spinco ultracentrifuge, and analyzed for nitrogen. The lung residue was autoclaved with water, centrifuged, and the extract *b* filtered through the Seitz filter and analyzed.

Extract *a* gave a precipitate with antiserum of rabbits no. 41 and 31 (see table 1), indicating the presence of the antigens, tuberculoprotein and possibly of tuberculopolysaccharide. Extract *b*, obtained after autoclaving, showed a precipitate only with antiserums of rabbits no. 17 and III6=51, but not with the antiprotein antiserum of rabbit no. 2, indicating the presence of tuberculopolysaccharide antigen, which probably remained as a hapten after autoclaving. Both extracts gave only a transparent crystalline precipitate with normal serum, which was obviously nonspecific and is not to be confused with the finely dispersed specific precipitates. Since this crystalline precipitate occurred only when fluids from heterologous animals were used, it is not concerned with these studies.

The presence of a small amount of antibody in extract *a* was also indicated by the precipitin reactions given with the antigens (table 1).

Isolation and identification of antigens and antibodies in the lungs of rabbit no. 41.—The lungs of rabbit no. 41, which had been sensitized by 15 weekly intracutaneous injections (0.25 mg.) of live BCG organisms, then infected intravenously with 0.0005 mg Ravenel strain of

bovine tubercle bacillus, and subsequently given 13 weekly injections (0.5 mg) of tuberculoprotein A, showed at death extensive caseation with practically no normal tissue left. Similar lesions were found previously in rabbits sensitized with tuberculoprotein.[6] The serum of this rabbit showed at the time of infection heavy precipitating antibodies for both tuberculoprotein and tuberculopolysaccharide, but after 4 injections of the tuberculoprotein, the antibodies to the polysaccharide disappeared, while those to the protein became heavier and remained so up to the time of death, which was only 140 days after inoculation. The lung of this rabbit weighed 65.6 g.

It was homogenized with about twice its weight of 0.9% saline* and allowed to extract overnight at 4 C. The supernatant, after centrifuging for 1 hour at 3000 r.p.m. at 4 C, was filtered through paper and then through the Seitz filter. It was called extract no. 1. The tissue residue was then stirred with 20% saline, incubated 2 hours at 37 C, and kept overnight at 4 C. A slimy ropy precipitate resulted which was difficult to separate from the liquid. By centrifugation and filtration an extract was obtained, which was then filtered through a Seitz filter. On dialysis against 0.9% saline a heavy precipitate occurred, some of which was not soluble again in 20% saline. The insoluble material was removed, and the soluble portion was again dialyzed against 0.9% saline. The fraction precipitating out was called extract no. 2. The residue from the 20% saline extraction of the tissue was then mixed with water and autoclaved. The supernatant from this extraction was then filtered through the Seitz filter and labelled extract no. 3.

The 0.9% saline extract (no. 1) showed the presence of antigen when tested with three different antiserums (table 1). That the precipitates were specific is shown by the fact that no precipitate was given with normal serum no. 84. This extract also contained considerable antibodies to tuberculoprotein, as shown in table 1, but no antibodies to tuberculopolysaccharide. This is consistent with the fact that the serum of this rabbit also had antibodies only to protein.

Neither antigens nor antibodies could be detected in the autoclaved extract no. 3. Extract no. 2 will be discussed in the section on antigen-antibody complexes.

Isolation and identification of antigens in the lungs of rabbit no. 54.—This rabbit was a control rabbit and had had no treatment prior to nor following intravenous inoculation with 0.0005 mg Ravenel strain of bovine tubercle bacillus. It died in 122 days with extensive tuberculosis. The lungs, however, were soft and weighed only 33.8 g. The lesions protruded in flower-like formation. Extracts with 0.9% saline (no. 1), 20% saline (no. 2), and after autoclaving (no. 3) were made in the same way as described for lung no. 41.

The presence of specific antigen was found in extract no. 1 when tested with three different antiserums, as shown in table 1, while no precipitate was given with normal serum no. 84. No antibodies were detected in either extracts no. 1 or no. 3. A precipitate, occurring on dialysis of the 20% saline extract and labelled no. 2, will be discussed under the section on antigen-antibody complexes.

Isolation and identification of antigens and antibodies in caseous material of rabbit no. 53.—The thick caseous material from two thick-walled cysts, weighing 56.7 g, found attached to the mesentery in the lower abdominal region of control

* All saline solutions used in these experiments contained 0.01% merthiolate as a preservative.

rabbit no. 53, was chosen for study of caseous material, since the rabbit died with extensive tuberculosis 94 days after intravenous injection of 0.0005 mg Ravenel strain of bovine tubercle bacilli. Its lungs were almost completely replaced with caseous material and it also had tubercles in the kidneys and spleen.

Extracts no. 1, 2 and 3 were prepared from this caseous material by the same methods as used for lung no. 41.

The presence of specific antigens was found in the 0.9% saline extract (no. 1) when tested against two antiserums of rabbits no. 41 and 49, while no precipitate occurred with normal serum no. 84. See table 1. Since the precipitate with antiserum from rabbit no. 41 was especially heavy, much tuberculoprotein must have been present. A small amount of antibody also appeared to be present when tests were made with specific antigens. The presence of specific antigens and possibly some antibodies would seem to furnish evidence that at least some of this caseous material was of tuberculous origin.

A fraction, no. 2, insoluble in 0.9% saline but soluble in 20% saline was also isolated, as in the case of the lungs of rabbits no. 41 and 54, and will be discussed under the following section.

Antigen-antibody complexes.—In this study the relative solubilities of the antigen-antibody precipitates in the various reagents were noted by reading the turbidities of their suspensions in a Klett colorimeter with filter λ660. These readings are recorded in table 2 as the numbers toward the right in each section. Quantitative studies of the solubilities will be made in the future by nitrogen analyses on more highly purified fractions.

The antigen-antibody precipitates formed in vitro, when tuberculoprotein or tuberculopolysaccharide was added to their antiserums, were first studied. These precipitates were insoluble in 0.9% saline, as was to be expected. The precipitate formed by tuberculopolysaccharide with BCG antiserum (no. III6 = 51)—the polysaccharide antigen-antibody complex—was completely soluble in excess polysaccharide, but insoluble in excess tuberculoprotein. It was partially soluble in 20% saline. The precipitate, however, was so compact that it was not possible to disperse it into a suspension sufficiently fine to read in the colorimeter. Therefore, no turbidity readings are recorded.

The precipitate formed by tuberculoprotein with antiserum no. 41—the protein antigen-antibody complex—was partially soluble in 20% saline, in excess tuberculoprotein, and also in excess

TABLE 2.—*Presence of antigen-antibody complexes.*

Tissue and rabbit numbers	Solubilities in:							
	0.9% NaCl	Klett R	20% NaCl	Klett R	Polysaccharide No. 1	Klett R	Tuberculoprotein (107 II)	Klett R
Serum antigen-antibody precipitates								
No. III6 = 51 with polysaccharide no. 1	Insoluble		Partially soluble		Completely soluble		Insoluble	
No. 41—with protein 107 II	Insoluble	21	Partially soluble	18	Partially soluble	10	Partially soluble	15
Crude suspensions								
Lungs No. 41—Milky	Insoluble	30	Partially soluble	27	Insoluble	32	Insoluble	31
Hard	Insoluble	23	Partially soluble	17	Insoluble	25	Partially soluble	19
Lungs no. 54	Insoluble	35			Partially soluble	30		
Caseous material no. 53	Insoluble	296	Partially soluble	231	Insoluble	299	Insoluble	310
Caseous material no. 46	Insoluble	18	Partially soluble	12	Partially soluble	17	Partially soluble	17
Isolated extracts no. 2								
Lungs no. 41	Insoluble	29	Soluble	0	Partially soluble	18	Partially soluble	9
Lungs no. 54	Insoluble	20	Soluble	0	Partially soluble	17	Partially soluble	13
Caseous material no. 53	Insoluble	4	Soluble	0	Partially soluble	2	Partially soluble	2

tuberculopolysaccharide. Its suspensions were very fine.

After determining that known specific antigen-antibody complexes were really soluble in antigen excess as well as in higher salt concentration, preliminary tests were made on crude extracts of caseous material found in the lungs or elsewhere in tuberculous rabbits.

Four crude extracts were made by macerating caseous material from lungs no. 41 and 54 and from caseous material from the sacs of rabbit no. 53 and from the caseous nodule of rabbit no. 46 in 0.9% saline, centrifuging off the larger clumps, and adding to aliquots of the milky suspensions (a) 0.9% saline, (b) 20% saline, (c) 2.5 mg tuberculopolysaccharide, and (d) 2.5 mg tuberculoprotein. These mixtures were incubated at 37 C for 30 minutes and placed in the cold room overnight. They were then made up to equivalent volumes and read in the colorimeter.

The results show (table 2) that in the crude suspensions of caseous lung material from rabbit no. 41 and in the suspension of caseous material from rabbits no. 53 and no. 46 some of the material dissolved in 20% saline. The hard caseous material of the lung of rabbit no. 41 and from rabbit no. 46 was also partially soluble in excess tuberculoprotein, and that from the lung of rabbit no. 54, and caseous nodule of rabbit no. 46 in excess tuberculopolysaccharide.

These solubility results suggested the possible presence of insoluble antigen-antibody complexes in caseous tissues. Such complexes may be soluble in high salt concentration, as was found by Heidelberger and coworkers[8,9] in the case of pneumococcus specific precipitates and albumin antigen-antibody complexes.

Such fractions, which were completely soluble in 20% saline, but insoluble in 0.9% saline, were isolated as described above, from the lungs of rabbits no. 41 and 54 and also from the caseous material of rabbit no. 53. The solubilities of these three fractions were studied, and table 2 shows that they were all partially soluble in excess tuberculopolysaccharide and in excess tuberculoprotein, as well as being completely soluble in 20% saline. A detailed study of the dissociated components of these complexes is in progress.

DISCUSSION

Antigens of the tubercle bacillus, and also antibodies to these antigens have been shown in this study to be present simultaneously in rabbit tissues. They were found in greatest concentration in the tuberculous lung tissue of a rabbit with massive caseation and also in other caseous material. Opie[10] believed that the simultaneous presence of antigen and antibody in the tissues resulted in local precipitation. Arthus and Breton[11] spoke about the "soft caseous material at the bottom of an ulcer" caused by the injection of horse serum into a previously sensitized rabbit. Taliaferro and

8. Heidelberger, M. and Kendall, F. E. 1936, Quantitative studies on antibody purification. I. The dissociation of precipitates formed by pneumococcus specific polysaccharides and homologous antibodies. J. Exper. Med. **64**: 161–72.

9. Heidelberger, M. and Kabat, E. A. 1938, Quantitative studies on antibody production. II. The dissociation of antibody from pneumococcus specific precipitates and specifically agglutinated pneumococci. J. Exper. Med. **67**: 181–99.

10. Opie, E. L. 1924, Acute inflammation caused by antibody in an animal previously treated with antigen. The relation of antigen to antibody in the Arthus phenomenon. J. Immunol. **9**: 255–68.

11. Arthus, M. and Breton, M. 1903, Lésions cutanées produites par les injections de sérum, de cheval Chez le lapin anaphylactisé par et pour ce sérum. Compt. rend. **55**: 1478–80.

Sarles[12] in their studies on immunity to *Nippostronglus muris*, showed clearly that "Antibodies (precipitins and possibly other humoral factors) immobilize, form precipitates in and around, stunt and sometimes kill the worms."

It is possible, therefore, that such antigen-antibody complexes are regularly contained in the caseous masses found in tuberculous tissue. In fact, a fraction was isolated from tuberculous lungs and other caseous material which was soluble in 20% saline but insoluble in 0.9% saline, and the solubility was reversible. The solution of this precipitate might be explained as a dissociation by strong salt of an antigen-antibody complex, such as had been found by Heidelberger and coworkers[8,9] in their studies on the immunology of the pneumococcus. However, the strongest evidence that these fractions contain such complexes is that they are soluble to some extent in excess antigen.

Our results show, furthermore, that an antigen-antibody complex made by precipitating BCG antiserum by tuberculopolysaccharide antigen, may be readily and completely dissolved in antigen excess, whereas the complex involving the tuberculoprotein antigen is much less soluble in its antigen excess. It was interesting that the precipitate of the antiprotein serum (rabbit no. 41) made by the tuberculoprotein could also be resolved to some extent by the addition of excess tuberculopolysaccharide. However, this is consistent with the previous finding[1] that the polysaccharide was able to inhibit the precipitation between protein antiserum and the protein, as well as the complement-fixation of PPD-S by this antiserum. The mechanism of this inhibition reaction is under investigation. The importance of the tuberculopolysaccharide in these immunological reactions should be emphasized, since in the region of polysaccharide excess no precipitate was obtained with antiserum known to contain specific antibodies for this antigen. Furthermore, the fact that this region of antigen excess begins with an extremely low concentration of polysaccharide (10 γ per 0.1 ml serum) focuses attention on the high immunological reactivity of this antigen. It is possible, therefore, that the presence of antibodies in serum may be masked by such an antigen.

It was found previously[1] that antibodies to the tuberculopolysaccharide were deficient in two rabbits of known genetic susceptibility to tuberculosis and in other rabbits unable to produce antibodies to polysaccharide, or in which these antibodies had been suppressed through the injection of excess tuberculoprotein or polysaccharide. In all such rabbits the more dissociable kind of antigen-antibody complex, that with the polysaccharide, would probably not be present to any extent. On the other hand, both dissociable and less dissociable types of complexes could be expected to be present in rabbits capable of forming both types of antibodies.

The findings with rabbit no. 41 were consistent with these expectations. It had received many injections of tuberculoprotein following infection and showed heavy anti-protein antibodies but no antipolysaccharide antibodies in its serum. Moreover, both antigen and antibody, and exclusively the antiprotein antibody, were demonstrated in its lung tissue. It died relatively soon,

12. Taliaferro, W. H. and Sarles, M. P. 1939, The cellular reactions in the skin, lungs and intestine of normal and immune rats after infection with *Nippostronglus muris*. J. Infect. Dis. **64**: 157–193.
——. 1942, The histopathology of the skin, lungs and intestine of rats during passive immunity to *Nippostrongylus muris*. J. Infect. dis. **71**: 69–82.

and at autopsy the lungs were almost completely filled with large solid caseous lesions and there was extensive tuberculosis throughout the body. Lurie[7] found that "caseous masses of tubercles in the susceptible animal containing numerous bacilli are compact and amorphous, whereas the corresponding foci in resistant rabbits undergo rapid and extensive liquefaction."

Rich[13] stated that caseation occurs in infections caused by microorganisms that attract mononuclears, as in tuberculosis, typhoid, syphilis and tularemia. It is also in this group of cells that the chief producers of antibodies have been found.[4,5] Moreover, monocytes[2] and plasma cells[3] and antibodies to tuberculoprotein[1] have been shown to increase following injections of tuberculoprotein. Further evidence that antibodies are involved in caseation is indicated by the fact mentioned by Rich[13] that "in certain desensitized animals which for reasons . . . lose their resistance, necrosis of tissue does not occur even though enormous numbers of bacilli are concentrated in the tissues." Furthermore, Yamamura and coworkers[14] were able to produce cavities by injecting heat killed bovine tubercle bacilli, suspended in paraffin oil and anhydrous lanoline, into the lung of rabbits, only if the rabbits had been previously sensitized by killed tubercle bacilli.

13. Rich, A. 1950, The Pathogenesis of Tuberculosis. Baltimore, C. C. Thomas, p. 742.
14. Yamamura, Y., Yasaka, S., Nakamura, S., Yamaguchi, M., Ogawa, Y., Endo, K. and Takeuchi, H. 1955, Experimental formation of tuberculous cavity by chemical constituents of tubercle bacilli. Proc. Japan Acad. **31**: 36–40; Med. J. Osaka Univ. **6**: 197–203

SUMMARY

Specific antibodies to tuberculo-protein and tuberculopolysaccharide were found in serum of rabbits repeatedly sensitized with BCG.

The antigen-antibody precipitate formed by adding tuberculopolysaccharide to BCG antiserum was completely soluble in excess polysaccharide.

The antigen-antibody precipitate formed by adding tuberculoprotein to antiprotein serum was partially soluble in excess tuberculoprotein, in excess polysaccharide, and also in 20% saline.

The simultaneous presence of both specific antibodies and specific antigens was found in tuberculous caseous lungs and in other caseous material.

Fractions were isolated from two tuberculous lungs and other caseous material which were insoluble in 0.9% saline but soluble in 20% saline. These fractions were shown to contain specific antigen-antibody complexes, since they were partially soluble in antigen excess.

It is suggested that such complexes formed in situ in tuberculous tissues could be resolved when the antigen concentration reaches the region of antigen excess. Such resolution could occur more rapidly in animals where the antibodies to polysaccharide are more abundant.

A SURVEY OF HEMOLYTIC STREPTOCOCCI FROM PATIENTS WITH RHEUMATIC FEVER, GLOMERULONEPHRITIS, PHARYNGITIS AND THE CARRIER STATE

ROBERT A. HARDIN, ROBERT W. QUINN, AND ROY C. AVERY

From the Department of Preventive Medicine and Public Health, Vanderbilt University, School of Medicine, Nashville 5, Tennessee

There is considerable evidence to support the view that in the pathogenesis of acute rheumatic fever and acute glomerulonephritis a preceding infection with beta hemolytic streptococci is necessary. However, only a very small percentage of hemolytic streptococcal infections are followed by these sequelae and the exact relationship is yet to be determined. Among the hypotheses of the pathogenesis of rheumatic fever is the one which would favor heredity in its occurrence,[1–3] namely, a fundamental difference in the host. Strong emphasis is placed on environmental factors by many investigators, and others suggest that certain strains of hemolytic streptococci may have rheumatogenic properties,[4] but, to date these strains or properties have not been identified. On the basis of recent epidemiologic observations,[5,6] it does appear, however, that there may well be "nephritogenic" strains of hemolytic streptococci. Many of the biological characteristics of hemolytic streptococci have been well-documented and there have been numerous studies of individual strains of hemolytic streptococci recovered from patients with one or another of the clinical manifestations of streptococcal infections. There have been no reports comparing biological characteristics of hemolytic streptococci isolated from groups of patients exhibiting manifestations of streptococcal infections or their complications.

It is the purpose of this paper to report the results of studies of some of the biological characteristics of certain strains* of hemolytic streptoccoci recovered from patients with streptococcal infections or complications of streptococcal infections, in an effort to determine if there might be any findings indicative of the so-called nephritogenic or rheumatogenic strains. The 86 strains from 80 individuals were isolated from 4 groups of patients with the following diagnoses: (1) acute rheumatic fever (throat cultures were obtained within 2 to 3 weeks from the time of onset and were assumed to be the strain responsible for the preceding sore throat); (2) acute glomerulonephritis (these throat cultures were also obtained within 2 to 3

Received for publication April 30, 1956.

1. Wilson, M. G. 1940, Rheumatic Fever, New York, The Commonwealth Fund.
2. Read, F. E. M., Ciocco, A. and Taussig, H. B. 1938, The frequency of rheumatic manifestations among the siblings, parents, uncles, aunts, and grandparents of rheumatic and control patients. Am. J. Hyg. **27**: 719–737.
3. Wilson, M. G., Schweitzer, H. D. and Lubschez, R. 1943, The familial epidemiology of rheumatic fever. I. Genetic studies. J. Pediat. **22**: 468–492.
4. Coburn, A. F. and Young, D. C. 1949, The Epidemiology of Hemolytic Streptococcus, The Williams and Wilkins Company.
5. Rammelkamp, C. H., Jr., Weaver, R. S. and Dingle, J. H. 1952, Significance of the epidemiological differences between acute nephritis and acute rheumatic fever. Tr. Assoc. Am. Phys. **65**: 168–175.
6. Rammelkamp, C. H., Jr., Stetson, C. A., Krause, R. N., Perry, W. D. and Koehn, R. J. 1954, Epidemic nephritis. Tr. Assoc. Am. Phys. **67**: 276–282.

* Sixty-seven of these strains were obtained from Dr. Floyd W. Denny, formerly of the Department of Pediatrics, Vanderbilt University, School of Medicine.

weeks from the time of onset and were assumed to be the organism responsible for the preceding streptococcal infection); (3) streptococcal pharyngitis without complications; and (4) streptococcal carriers who had the same group and type of hemolytic streptococci in their throat for several months without any clinical manifestations.

METHODS AND MATERIALS

The streptococci from each patient were isolated in pure culture and then the following characteristics were determined for each strain:

1. Group and type.
2. Reduction of methylene blue.
3. Carbohydrate fermentation. (a) trehalose, (b) sorbitol, (c) glycerol, (d) mannitol, (e) lactose, (f) sucrose, (g) raffinose, (h) salicin, (i) inulin.
4. Ability to liquefy gelatin.
5. Ability to resist heating at 60 C for 30 minutes.
6. Final pH in 1% glucose broth.

The usual methods of testing for the above characteristics were employed and then each strain was tested for its ability to produce:

7. Streptolysin O
8. Streptolysin S
9. Streptokinase
10. Hyaluronic acid
11. Hyaluronidase
12. Proteinase
13. Erythrogenic toxin

Streptolysin O.—Cultures were grown for 18 hours at 37 C in Todd-Hewitt broth. The streptolysin was reduced just prior to use by the addition of 1.5 mg. per ml of L cysteine hydrochloride. The test was performed as shown in table 1. The appropriate amount of streptolysin was made up to 1.5 ml by the addition of normal saline. Five-tenths ml of 5% rabbit erythrocytes in *M*/15 phosphate buffer in normal saline pH 7.2, was added to each tube. The tubes were shaken vigorously, incubated at 37 C for 1 hour, centrifuged and the results read. The last tube, reading from tube 1, in which hemolysis appeared, was taken as the end point. If the hemolytic end point was seen in tube number 1, that particular strain arbitrarily was recorded as producing streptolysin O in an amount of 1+, tube number 2, 2+ etc.

Streptolysin S.—The method employed for the determination of streptolysin S was adapted from that of Weld.[7] Yeast ribonucleic acid was added because the work of Bernheimer and Rodbart[8] indicated an increase in production of streptolysin S after the addition of this material. Cultures were incubated at 37 C for 13 hours. The tubes were thoroughly chilled and centrifuged until the supernatant was clear. The supernatant was discarded and 0.5 ml of horse serum, pH approximately 8.0, inactivated at 56 C for 40 minutes, was added. Sterile glass beads were added and the tubes were shaken in a shaking machine for one hour. Tubes were thoroughly chilled and centrifuged. The following dilutions of the serum extract were made in *M*/15 phosphate buffer, pH 7.2; 1:2, 1:10, 1:100, 1:500, 1:1000, and 1:2000. To each tube was added 1.0 ml. of a 1% suspension of washed rabbit erythrocytes. The tubes were then incubated in a water bath at 37 C for 1 hour, and after centrifuging were observed for hemolysis. Results were recorded as the greatest dilution of serum extract showing hemolysis.

Streptokinase.—Eighteen-hour cultures in beef heart infusion broth containing 1% dextrose and 0.001% phenol red were tested by the fibrin clot lysis method of Kaplan et al.[9] Cultures were neutralized frequently during the last 8 hours of growth with 1 *N* NaOH to a pH of approximately 7.0. The tubes were centrifuged until the supernates were clear, and the test was performed as shown in table 2. The tubes were observed for

7. Weld, J. T. 1934, The toxic properties of serum extracts of hemolytic streptococci. J. Exper. Med. **59**: 83–95.
8. Bernheimer, A. W. and Rodbart, M. 1948, The effect of nucleic acids and of carbohydrates on the formation of streptolysin. J. Exper. Med. **88**: 149–168.
9. Kaplan, N. H. 1946, Studies of streptococcal fibrinolysis. III. A quantitative method for the estimation of serum antifibrinolysin. J. Clin. Investigation, **25**: 346–351.

TABLE 1.—*Procedure for the determination of streptolysin O.*

Tube no.	1	2	3	4	5	6
Streptolysin O ml/tube	0.5	0.4	0.3	0.2	0.1	0.0
Normal saline ml/tube	1.0	1.1	1.2	1.3	1.4	1.5
5% washed rabbit r.b.c. in buffered saline pH 7.2, 0.5 ml/tube	0.5	0.5	0.5	0.5	0.5	0.5
Results	1+	2+	3+	4+	5+	

TABLE 2.—*Procedure for the determination of streptokinase.*

Dilution of streptokinase (in phosphate buffer, pH 7.35)	Undiluted	1:4	1:16	1:64	1:128
Fibrinogen (3.3 mg/ml of phosphate buffer)	0.5 ml/tube				
Thrombin (1:10 in phosphate buffer)	0.5 ml/tube				
	0.1 ml/tube				

lysis after 30 minutes incubation at 37 C. The results were expressed as the highest dilution which showed complete lysis. When lysis was observed, the clot was nearly always completely lysed.

Hyaluronic acid.—A semiquantitative method described by Seastone[10] was used to test for hyaluronic acid. The growth medium was beef heart infusion (Difco) plus 10% sterile sheep serum. After 24 hours incubation at 37 C, 2 drops of 0.2 phenol red were added to each tube and the culture brought to about pH 7.6 with 0.5 *M* NaOH. Serial dilutions of the clear supernatant were made in 0.85% NaCl as follows: undiluted, 1:10, 1:20, 1:40, 1:80. These were taken up into microprecipitin tubes over acidified normal horse serum and readings were made after one-half hour. The formation of a precipitate at the interface indicated the presence of hyaluronic acid. Uninoculated mediums gave no ring at the interface.

Hyaluronidase.—The method for the determination of hyaluronidase was based on the mucin-clot prevention technique of McClean[11] as modified by Quinn.[13] Failure of clot formation indicated the presence of hyaluronidase. The results were expressed in the final dilution of culture supernate which prevented clot formation.

Proteinase.—The method described by Elliot and Dole[13] was employed for the determination of proteinase. The growth medium was beef heart infusion broth (Difco) plus 2.0% bacto-peptone and 0.2% dextrose. Strains were grown for 24 hours at 37 C and dilutions of 1:10 and 1:100 of the supernate were made in phosphate buffer pH 7.0. Three-tenths ml aliquots of the diluted culture supernate and milk-thioglycolate mixture were mixed in agglutination tubes and incubated in a water bath for 18 hours at 37 C. It is important that the entire procedure be performed using sterile equipment and sterile technique. Coagulation of the milk was interpreted as an indication of the presence of proteinase.

Erythrogenic toxin.—The method used was an adaptation of the methods described by Wadsworth[14] and Smith.[15] The medium employed was beef heart infusion (Difco) with 2.0% proteose peptone (Difco). Cultures were grown for 24 hours at 37 C and then allowed to stand at room temperature for 14 hours. The supernatants were placed in sterile tubes and heated in a water bath for 30 minutes at 56 C. The tubes were then stored at 2 C until tested. Dilutions of 1:10 and 1:100 were made in physiological saline and 0.1 cc injected intradermally into a white female goat aged 18 months. Skin reactions were read at 18 hours. The slightest erythema about the injection site of either dilution was recorded as a positive reaction indicating the presence of erythrogenic toxins since sterile medium gave no erythema.

RESULTS

The results are recorded in tables 3, 4, and 5. All of the strains were β hemolytic and group A with the exception of one which could not be grouped. Slightly over half were not typeable with the available typing serums.* With very few exceptions the results of the reduction of methylene blue, fermentation of

10. Seastone, C. V. 1943, The occurrence of mucoid polysaccharide in hemolytic streptococci of human origin. J. Exper. Med. **77**: 21–28.
11. McClean, D. 1943, Studies in diffusing factors II. Methods of assay of hyaluronidase and their correlation with skin diffusing activity. Biochem. J. **37**: 169–177.
12. Quinn, R. W. 1948, Studies of the mucin-clot prevention test for the determination of the antihyaluronidase titre of human serum J. Clin. Investigation, **27**: 463–470.
13. Elliot, S. D. and Dole, V. P. 1947, An inactive precursor of streptococcal proteinase. J. Exper. Med. **85**: 305–320.
14. Wadsworth, A. B. 1947, Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, Baltimore, Williams and Wilkins Company.
15. Smith, J. 1927, The exotoxins of the hemolytic streptococci. J. Path. & Bact. **30**: 651–666.

* Groups A, B, C, D, G. Types 1, 2, 3, 4, 5, 6, 8, 11, 12, 13, 14, 15, 17, 18, 19, 22, 23, 24, 25, 26, 28, 29, 30, 31, 32, 33, 36, 37, 38, 39, 40, 41, 42, 43, 44, 46, and 47. The grouping and typing sera were generously supplied by Dr. Elaine L. Updyke, Communicable Disease Center, Chamblee, Georgia.

TABLE 3.—*Number of strains according to group and type.*

Group and Type	Nephritics	Rheumatics	Pharyngitis	Carriers	Total
A 1			2	3	5
A 3				1	1
A 4	1	2			3
A 5			1		1
A 6	1	2	5	2	10
A 12	13			1	14
A 19		2			2
A NT	11	18	17	3	49
NG		1			1
Total	26	25	25	10	86

NT, non-typeable; NG, non-groupable.

the various carbohydrates, liquefaction of gelatin, ability to resist 60 C for 30 minutes and the final pH were the same for all of the strains recovered from each of the four clinical syndromes regardless of the serological type, and were the same as those listed by Swift in Dubos[16] for group A streptococci.

The typeable, nontypeable and non-groupable strains are listed in table 3. It is noteworthy that group A, type 12, which frequently has been associated with acute glomerulonephritis, was recovered from 13 of the 26 patients with this diagnosis, types 4, 6 and no type being isolated from the remaining thirteen. In none of the other groups of patients was any type predominant although nontypeable strains were in heavy majority. These findings indicate that a variety of types of group A can and did cause pharyngitis and upper respiratory infections which preceded rheumatic fever and glomerulonephritis. It should be made clear that the carrier streptococci were not selected at random, but were picked to be representative of the types available from carriers.

In table 4, are listed the extracellular components and enzymes produced by each of the typeable and nontypeable strains of group A streptococci. There were no signficant differences among the different types in their production of any of the substances measured, nor between the typeable and non-typeable strains with the exception of proteinase which was elaborated four times as frequently by nontypeable strains.

In table 5 the bacteria are grouped according to the four clinical patterns with which they were associated. There were differences when the enzyme production of the strains associated with rheumatic fever were compared with nephritis and indeed there were differences in enzyme production among the bacteria recovered from each of the four clinical syndromes, but it is felt that these differences were not biologically specific for any of the streptococcal diseases even though statistical significance can be attached to some of the variations.

In general, enzyme production of specific types was the same qualitatively but there were striking differences in the quantity produced and in several instances the same type recovered from

16. Dubos, R. J. 1952, Bacterial and Mycotic Infections of Man, Philadelphia, J. B. Lippincott Company.

TABLE 4.—*Relationship of substances produced to group and type.*

Group and type	Streptolysin O	Streptolysin S	Streptokinase	Hyaluronic acid	Hyaluronidase	Proteinase	Erythrogenic toxin	Total
A 1	3	5	3	5	0	1	3	5
A 3	1	1	1	1	0	0	1	1
A 4	2	3	3	3	3	1	1	3
A 5	1	0	1	0	0	0	1	1
A 6	9	9	10	10	0	1	7	10
A 12	12	11	14	14	0	0	12	14
A 19	2	2	2	2	0	1	2	2
A NT	31	44	38	43	7	17	31	49
NG	1	0	0	1	0	0	0	1
Total	62	75	72	79	10	21	58	86

TABLE 5.—*Enzyme production of strains associated with clinical syndromes.*

Substance produced	Nephritics	Rheumatics	Pharyngitis	Carriers	Total
Streptolysin O	21	17	16	7	61
Streptolysin S	19	23	25	10	76
Streptokinase	26	19	20	7	72
Hyaluronic acid	26	22	21	6	75
Hyaluronidase	1	6	3	0	10
Proteinase*	3	9	7	2	21
Erythrogenic toxin†	22	11	14	6	53
Total number of strains in each group that were tested	26	25	25	10	86

* The following strains were not tested for proteinase production: 1 nephritic, 4 rheumatics, and 8 pharyngitis.
† Erythrogenic Toxin production was not determined for: 2 rheumatics, 2 pharyngitis, and 1 carrier.

different patients or on one or two occasions from the same patient but at different times, exhibited marked differences. For example, of three type 1's from three different carriers, one did not produce streptolysin O, two did not produce streptokinase, and two did not produce proteinase. Fourteen strains of type 12 were alike in enzyme production except for three which did not produce streptolysin S and two which failed to elaborate erythrogenic toxin and streptolysin O. Inconsistencies were noted in type 6, one of which produced no streptolysin O, one no streptolysin S and three no erythrogenic toxin. Two type 4 streptococci recovered from a patient with rheumatic fever approximately a week apart differed markedly in the quantity of streptolysin S produced and one produced both streptolysin O and proteinase while the other elaborated neither. Hyaluronidase production was limited to the three strains of type 4 and seven non-typeable.

In the original work of Elliot[17] it was suggested that the production of M protein and proteinase were mutually exclusive and the presence of proteinase may interfere with or make it impossible to type certain strains of group A streptococci. Later however, Rothbard and Todd[18] showed that the ability to produce streptococcal proteinase appeared to be related to the strain and not necessarily to the serological type and that many typeable strains which produced M protein also produced proteinase. They also obtained data which indicated that proteinase or non-proteinase-producing strains of various serological types were not associated with any particular variety of infectious process. The results of the present experiment tend to corroborate those of Rothbard and Todd because four typeable, M-producing strains also elaborated proteinase and proteinase producers were recovered from patients in all four of the clinical categories. It is significant though, that non-typeable streptococci produced proteinase four times as frequently as those which were typeable.

Practically all streptococci associated with scarlet fever form erythrogenic toxins,[19] but of those isolated from the four patient groups, only 67.4 percent formed erythrogenic toxin. Forty-six and six-tenths percent of these were typeable, 53.4% non-typeable. Significantly more of the nephritic strains were erythrogenic toxin producers than was the case with strains from any of the

17. Elliot, S. D. 1945, A proteolytic enzyme produced by group A streptococci with special reference to its effect on the type-specific M antigen. J. Exper. Med. **81**: 573–592.
18. Rothbard, S. and Todd, E. W. 1948, Production of Proteinase by hemolytic streptococci in various clinical conditions. J. Exper. Med. **87**: 283–294.
19. Coffey, J. M. 1938, Further observations on the toxigenic properties of hemolytic streptococci. J. Immunol. **35**: 121–130.

other clinical syndromes, although none of the patients had scarlet fever.

DISCUSSION

Perhaps it is too much to expect to demonstrate in vitro biological differences in bacteria, which might explain why rheumatic fever or nephritis occurs after infection with certain types. In vitro measurements probably resemble remotely, if at all, the actual biological behavior of these bacteria in the host and then too it may not be specific strains of the infectious organisms at all which are responsible for certain complications of streptococcal infections, there may be certain characteristics of the host which render him susceptible to rheumatic fever. Bearing in mind the short comings of in vitro determinations, these studies furnish no evidence of the association of any specific type of group A hemolytic streptococcus with patients with rheumatic fever. A variety of typeable and non-typeable strains were recovered from these patients and none of them exhibited any special biologic characteristic which might in any way set it apart as a so-called rheumatogenic streptococcus. In other words it would appear that any group A streptococcus could cause an upper respiratory infection which might be followed by rheumatic fever.

The recovery of 13 type 12 strains from 26 patients with acute glomerulonephritis certainly suggests that there may be some characteristic of this type which renders it more nephritogenic than other types but these studies failed to reveal this characteristic. If there is a nephritogenic substance, other serological types of streptococci must elaborate it too since 11 non-typeable, one type 4 and one type 6 also were isolated from patients with acute glomerulonephritis. It should be emphasized too that these 26 patients admitted to the pediatric service of Vanderbilt Hospital were probably only a small percentage of the cases occurring in the Middle Tennessee area and no information is available concerning the types of streptococci associated with the cases which were not admitted.

SUMMARY AND CONCLUSIONS

Eighty-six strains of β hemolytic streptococci were isolated from eighty patients. Twenty-five were from patients with acute rheumatic fever, 26, 13 of which were type 12, from acute glomerulonephritis, 25 from acute pharyngitis and 10 from carriers who were not clinically ill. Each strain was grouped and typed and tested for well-known characteristics listed previously. These results were remarkably similar for each strain and identical with those usually associated with group A streptococci. In addition, determinations were made for streptolysin O, streptolysin S, streptokinase, hyaluronic acid, hyaluronidase, proteinase and erythrogenic toxin. There were striking differences both qualitatively and quantitatively in the elaboration of these substances by many of the strains and occasionally by strains of the same type. No pattern of enzyme production characteristic of any typeable or non-typeable strain or of those strains recovered from patients with any of the four clinical syndromes could be demonstrated, nor could any "rheumatogenic" or "nephritogenic" substance be related to their cultural, enzymatic, or serologic characteristics.

STUDIES ON IMMUNITY TO ASIATIC CHOLERA

VIII. THE VIRULENCE OF STRAINS OF *VIBRIO CHOLERAE* FOR THE MOUSE

SYED S. HUSAIN AND WILLIAM BURROWS

From the Department of Microbiology, The University of Chicago, Chicago 37, Illinois

The virulence of *Vibrio cholerae*, like that of many similar pathogenic microorganisms, is as yet a highly uncertain quality. While there is adequate reason to believe that isolates of the culturally and serologically "typical" vibrio from clinically characteristic cases of the disease are capable of causing cholera in man, viz., laboratory infections including the famous human experiment of von Pettenkofer, there is as yet no laboratory procedure which will allow the identification of virulence in these microorganisms.

Virulence for man is an essential feature of the definition of this microorganism. The isolation of culturally typical "agglutinable" vibrios under circumstances in which the human disease does not occur leads to the belief that its characterization is inadequate. Conversely, the identification of the Celebes vibrio (de Moor, 1938), differing from *V. cholerae* in that it is hemolytic by the Greig test, as the etiologic agent of epidemic diarrheal disease in the Netherlands East Indies that was indistinguishable from Asiatic cholera on the one hand, the common occurrence of closely similar, if not identical, El Tor vibrios of serological O group I in the absence of diarrheal disease (Doorenbos, 1938) on the other, also suggest that the essential features of the etiologic agent are as yet not fully defined. It is possible that the disease Asiatic cholera may be caused by more than one variety of the genus Vibrio but not by still others, but such hypothetical differences in virulence lack substantiation.

Received for publication May 7, 1956.

The epidemiological evidence (Swaroop and Pollitzer, 1955) is taken by some to suggest that at least in some areas, such as the Ganges Delta, the case is not an immediately important source of epidemic infection. The source of infection in the epidemic disease frequently appears to be contaminated water, but the origin of the contamination is not clearly defined nor, in fact, do the epidemic periods invariably coincide with peaks in the numbers of agglutinable vibrios found in the river waters (Krishnan, personal communication). It might be inferred that even a short sojourn in the human intestinal tract suffices to give rise to avirulent variants of the vibrios, and some workers (Pandit and Hora, 1951) have suggested the hilsa fish constitutes an animal reservior of the virulent microorganism that is favored by the reduced salinity of the Bay of Bengal. Such possibilities necessarily remain purely speculative in the absence of criteria for virulence, and therefore precise characterization, of *V. cholerae*.

Probably the best working definition of *V. cholerae* is that of a morphologically, culturally and serologically typical microorganism isolated in pure culture, or substantially so, from the rice water stool of a patient with the clinically typical disease. The last term requires modification for in the experience of one of us (W. B.) "typical" clinical cholera is closely simulated in undernourished individuals by severe diarrheal disease of other than *V. cholerae* etiology. The typical human case of the disease, then, should occur during an epidemic of cholera rather than in an interepidemic period.

Virulence for man is commonly, and no doubt sometimes questionably, inferred from the demonstrable virulence of the microorganism for a laboratory animal. In 1885 Koch reported enteric infection of the guinea pig achieved by intragastric inoculation of *V. cholerae* culture. This kind of infection was subsequently studied by Cantacuzene and Marie (1914), Sanarelli (1921), and most recently by Burrows, Elliott and Havens (1947), Freter (1955b) and Dutta and Habbu (1955), but has not been applied to the assay of the relative virulence of strains of *V. cholerae*. The mouse test introduced by Griffitts (1942) and used by Ranta and Dolman (1943) and Burrows, Mather, Elliott and Havens (1947) is an adaptation of the method used for the assay of the virulence of *Salmonella typhi* (Siler et al, 1941) and consists of the intraperitoneal inoculation of mice with graded doses of the microorganisms suspended in 5% mucin. The resulting fatal infection is a fulminating bacteremia, quite unlike the localized enteric infection of human cholera. While marked dissimilarity between the experimental and naturally occurring infections may not be significant as in the case of intracerebral versus intranasal (Pittman, 1951) or inhalation (Fishel, 1955) challenge inoculation with *Hemophilus pertussis* in the mouse, it is difficult to reconcile the fulminating bacteremia in the mouse and the essentially local infection confined to the lumen of the bowel in man produced by *V. cholerae* with the same mechanisms of microbial pathogenicity. Nevertheless, the mouse test has been widely applied in both the assay of virulence of *V. cholerae* and of immunogenic potency of cholera vaccines. This test does not appear to have been studied systematically in an attempt to correlate human and mouse virulence, and such a study is the subject of the present report.

MATERIALS AND METHODS

Vibrio strains.—An essential preliminary to any attempt to assay the virulence of *V. cholerae* for man as expressed or reflected in behavior under experimental conditions is the availability of a group of strains of the vibrio with precisely known histories from which some assumption of human virulence may be hypothecated. To this end one of us (W.B.)* studied a group of 48 cases of typical clinical cholera admitted to the Nilratan Sarkar Medical College Hospital (Campbell Hospital) in Calcutta during February and March of 1953. This period represented the early portion of the annual spring epidemic in Bengal in that year; at the beginning of the study 2 to 4 cases, diagnosed on clinical grounds as cholera, were admitted per week, and when the study was concluded the admission rate was approximately 40 per day.

Of these 48 cases, one clinically typical case yielded a pure culture of culturally characteristic but inagglutinable vibrios and was discarded from present consideration. The remainder may be regarded as bacteriologically confirmed in so far as the cultural and serological character of the isolates was concerned. Six of the confirmed cases were fatal; 5 died within 24 hours of admission, while one relapsed and died on the 4th day. In 43 or almost 90% of the cases, the first culture was taken within 30 hours of onset of symptoms; in 20 or 42.5%, the first culture was taken within 18 hours; and in 6 cases the interval was 6 hours or less, including one taken at 3 hours and one at 4 hours after onset. In 2 cases the time of onset could not be ascertained. No patient received chemotherapy during hospitalization, and only one had received sulfaguanidine prior to admission. Stool specimens were cultured every day until death or discharge from the hospital, and a

* In collaboration with Dr. C. E. Lankford of the University of Texas. This study was made possible by the cooperation of Dr. K. V. Krishnan, Director of the All-India Institute for Hygiene and Public Health who made laboratory facilities available, and that of Drs. M. N. Lahiri and P. N. Bose of the staff of the Institute, and of Dr. A. Mondal of the infectious disease section of the Nilratan Sarkar Medical College Hospital. The work was also facilitated by the active interest of Dr. K. V. Venkatraman of the Calcutta School of Tropical Medicine. Dr. W. M. Bonné and Dr. R. Pollitzer of the Division of Communicable Disease Control of the World Health Organization, and Dr. C. G. Pandit, secretary of the Indian Medical Research Council were of invaluable assistance in planning the study.

group of 135 isolates was obtained which represented all stages of the disease. No attempt was made to isolate *V. cholerae* other than by direct culture; enrichment methods for isolation of vibrios present only in small numbers would no doubt have given positive cultures over periods longer than the maximum of 6 days observed here.

The vibrio strains were serologically typed on isolation with monospecific Inaba, Ogawa and R antiserums supplied by the Central Research Institute, Kasauli, as well as with antiserums prepared at the University of Chicago and the University of Texas. With one exception all isolates were of the Ogawa serotype. Strains reacting with R antiserum were found occasionally after the 3rd day of the disease. On subsequent study all strains were found to be gram-negative vibrios, Heiberg fermentative type I, and negative to the Greig test for hemolysis, thus conforming to the usual definition of *V. cholerae*. The cultures were maintained in the frozen state as suspensions in 1% peptone water at −70 C, and as used in this study represented the 5th transplant from the original isolation plates. Certain of these strains have been used by other workers, by Gallut (1954) and Freter (1956) in studies on endotoxin, and by Freter (1955a, 1955b) in studies on the serological character of vibrio mucinases and on experimental enteric infection of the guinea pig. The strains used in the work reported here are shown in table 1.

Colonial morphology.—Prior to the isolation of the vibrio strains just described, the colonial morphology of stock strains of *V. cholerae* was studied by examination under oblique transmitted light (Henry, 1933) using the dissection microscope. A variety of colonial types were apparent which could be resolved into 4 general types, viz., (a) a minute translucent achromatic colony about 1 mm in diameter; (b) a closely similar type distinguished by friability and a roughened surface that gave a cut-glass appearance; (c) a dense, raised, white form 1 to 3 mm in diameter; and (d) a granular form 1 to 4 mm in diameter showing a variable (from strain to strain) degree of irregular concentric striation and characterized by a chromatic appearance varying from bronze to green from the top to the bottom of the individual colony. The distinctiveness of these types varied from one medium to another. They were sharply differentiated on "2-1" agar (2% glucose and 1% glycerol in NaCl nutrient agar), and relatively indistinct on peptone water agar, while on veal infusion agar all tended to assume the dense form. The cut-glass type appeared to be the most stable, but all were relatively unstable on all mediums and especially on McConkey's agar. A thionin-glycerol agar (1% Bacto peptone, 0.2% glycerol, 1.5% agar, thionin* to a concentration of 10 μg per 100 ml, and adjusted to pH 8.0) was developed which allowed maximal differentiation of the colonial types, prolonged viability not obtainable with "2-1" agar, and a high degree of stability of colonial type on successive subculture. This medium was not inhibitory in that it gave substantially the same plate counts as blood agar and peptone water agar.

A group of 103 stock vibrio strains of varied origin, including Indian strains received through the National Institutes of Health in 1942, El Tor strains of O group I, strains isolated during the Chungking and Egyptian epidemics, strains from Japan and Indochina and from Bengal, were cultured on thionin-glycerol agar and examined for colonial morphology. Of these, 26 were the translucent type, 18 the cut-glass type, 35 the dense type, and 24 the granular chromatic type. No colonial type appeared to be associated with El Tor vibrios in contrast to the strains of *V. cholerae*.

When the Calcutta group of *V. cholerae* strains described above was isolated 3 mediums were inoculated with rice water stools or rectal swab specimens. These were the bile salt agar (NaCl nutrient agar containing 0.5% sodium taurocholate and adjusted to pH 8.0) routinely used for the isolation of cholera vibrios by workers in Calcutta, "2-1" medium, and the thionin-glycerol medium just described. It was of particular interest to find that of the colonial types demonstrable on the last two mediums, only the granular chromatic type occurred on the primary isolation plates; there appeared to be no relation between the degree of striation and the history of the isolate. It seemed probable, then, that colonial types other than this may be regarded tentatively as variant types of uncertain relation to the human disease. Only vibrio cultures showing this colonial type were used in the present study.

Animal inoculation.—The inoculum was prepared from frozen cultures grown on thionin-glycerol agar for 24 hours, and harvested in chilled physiological salt solution; it represented the 6th transplant from the original isolation plates. Ten-fold dilutions were prepared in chilled 5% sterile gastric mucin, held at 2 C until used, and immediately after animal inoculation was completed, viable counts of the 3 highest dilutions were made by pour plate using thionin-glycerol agar. It may be noted in passing that the

* Acid fuchsin, eosinate of methylene blue, or malachite green may be used in place of thionin but gave a slightly reduced differential effect.

TABLE 1.—*Source and character of* V. cholerae *strains isolated at Campbell Hospital, Calcutta in 1953.*

Case no.	Strain no.	Date	Hours after onset	Agglutination					Outcome of disease
				In saline	In antiserum to:				
					O Group I	Inaba	Ogawa	R	
20	21	2/17	11	−	+	+	+	−	
	22	2/18	35	−	+	+	+	−	
	23	2/19	59	−	+	+	+	−	
	24	2/20	83	−	+	+	+	−	Recovered
40	41	2/22	22	−	+	±	+	−	Recovered
60	61	2/22	8	−	+	±	+	−	
	62	2/23	32	−	+	±	+	−	Recovered
80	81	2/22	18	−	+	±	+	−	
	82	2/23	44	−	+	±	+	−	Recovered
100	101	2/23	27	−	+	±	+	−	Recovered
130	131	2/24	30	−	+	±	+	−	
	132	2/25	54	−	+	±	+	−	
	133	2/26	78	−	+	−	+	−	
	134	2/27	102	−	+	±	+	−	Recovered
140	141	2/24	32	−	+	±	+	−	
	142	2/25	56	−	+	±	+	−	
	143	2/26	80	−	+	±	+	−	
	144	2/27	86	−	+	±	+	−	Died 2/28
160	161	2/25	12	−	+	−	+	−	Recovered
170	171	2/26	16	−	+	±	+	−	
	172	2/27	40	−	+	±	+	−	
	173	2/28	64	−	+	±	+	−	
	173	3/1	88	−	+	±	+	−	
	175	3/2	112	−	+	±	+	−	Recovered
280	241a	3/1	10	−	+	−	+	−	
	241b	3/1	10	−	+	−	+	−	Recovered
250	251	3/1	17	−	+	+	±	−	
	252	3/2	41	−	+	−	+	−	Recovered
320	321	3/3	6	−	+	+	+	−	
	322	3/4	30	−	+	−	+	−	
	323	3/5	54	−		−	+	−	
	324	3/6	78	−	+	+	+	+	
	325	3/7	102	−	+	−	+	−	Recovered
340	341	3/3	26	−	+	−	+	−	Recovered
370	371	3/4	9	−	+	+	+	−	
	372	3/5	33	−	+	+	+	−	Died 3/5
380	381	3/4	?	−	+	+	+	−	
	383	3/6	?	−	+	+	+	−	
	385	3/8	?	+	+	+	+	+	
	386	3/9	?	−	+	+	+	−	Recovered
400	401	3/5	6	−	+	+	−	±	Died 3/6
410	411	3/6	6	−	+	−	+	−	
	412	3/7	30	−	+	−	+	−	
	414	3/8	78	+	+	+	+	+	
	415a	3/8	102	+	+	+	+	+	
	415c	3/9	102	+	+	+	+	+	
	416a	3/10	126	+	+	+	+	+	
	416b	3/10	126	+	+	+	+	+	
	416c	3/10	126	+	+	+	+	+	
	416d	3/10	126	+	+	+	+	+	
	416e	3/10	126	+	+	+	+	+	
	418f	3/10	126	+	+	+	+	+	Recovered
440	441	3/6	19	−	+	±	+	−	Died 3/7
480	481	3/6	12	−	+	±	+	−	
	482	3/7	36	±	+	±	+	+	Recovered

amount of mucin present was sufficient to cloud the medium, and colonies of the microorganisms were surrounded by clear zones, presumably a consequence of mucinase activity. A similar phenomenon has been noted by others (Cherry, personal communication).

The mice used were a white, pen-bred strain previously shown to be of uniform susceptibility to infection with *V. cholerae*. The mice, 12 to 14 grams in weight, were inoculated intraperitoneally in groups of 4 with 0.5 ml of the serially diluted inoculum. Most deaths occurred in 24 to

48 hours, and the period of observation was arbitrarily limited to 72 hours in the LD_{50} titrations. The LD_{50} of viable microorganisms as shown by plate count was interpolated by the Reed and Meunch method.

In addition to the determination of the conventional LD_{50} as an expression of virulence, the developing bacteremia was followed in some groups of infected animals by periodic quantitative blood culture. Groups of 8 animals were used. The inoculums were varied, but the highest dilutions were sufficient to give approximately 100 % mortality except in the case of the avirulent R strains. Blood was taken 2 hours after inoculation and at intervals of 2 hours thereafter up to 8 hours; after 8 hours the interval was extended to 4 to 6 hours to the end of the total observation period of 16 to 20 hours when no deaths occurred. Most of the experiments were of shorter duration since no specimens were taken after the animals began to die because of the selective effect of survival.

Specimens were taken by cardiac puncture, using an 0.25 ml syringe and ½ inch 24-gauge needle, from anesthetized sacrificed animals. Tenfold dilutions of the 0.1 ml specimen were made in chilled 1% citrate solution (pH 7.3), and plates of thionin-glycerol agar inoculated by spreading the inoculums over the surface of the medium. After 24 hours incubation the plates were examined and counted. The cultures were almost always pure cultures, and in no instance was the colonial morphology other than identical with that of the culture used for animal inoculation and observed on primary isolation.

RESULTS

It might be supposed that the strains of *V. cholerae* having the greatest virulence for man would be isolated from fatal cases of the disease dying within 24 to 48 hours of onset of symptoms, while those from nonfatal cases would be of lesser virulence. Of those strains in the second group, it might be argued that the infection in those persons giving only 1 or 2 positive cultures was least virulent since it was eliminated so rapidly, or, conversely, that the least virulence for man was indicated by persistence of the microorganisms in persons showing clinical recovery. However this may be, uniformity of the host with respect to some degree of pre-existing immunity and rapidity of an effective immune response and/or resistance to infection of other than an immunological character, is implicit in any such assumptions. While individual differences may be washed out in large series of cases, it is unlikely that they can be disregarded in a series consisting of only 15 recovered cases and 4 fatal cases such as that studied here. Nevertheless, if there were large differences in mouse virulence between strains isolated from fatal and nonfatal cases, or isolated on the first day of the disease in contract with the 5th or 6th day, some indication of such differences might be found even in a small series.

Table 2 shows the mouse virulence of isolates as the conventional LD_{50}, with the cases grouped according to the above considerations. It is clear from these data that there was no marked

TABLE 2.—*Virulence of strains of* V. cholerae.

Outcome of disease	Case no.	Days of positive culture	Strain no.	LD_{50}
Fatal	400	1	401	536
	440	1	441	122
	370	2	371	45
			372	38
	140	4	141	300
			142	420
			143	189
			144	50
Nonfatal	40	1	41	550
	100	1	101	7,600
	160	1	161	325
	240	1	241a	250
			241b	280
	340	1	341	219
	60	2	61	132
			62	162
	250	2	251	125
			252	140
	480	2	481	500
			482R	3,050,000
	130	4	131	50,011
			132	49,000
			133	127,000
			134	16,500
	320		321	125
			322	114
			323	447
			324	78
			325	112
	380	6	381	93
			383	136
			383R	1,030,000
			386	95
	410	6	411	119,000
			412	57,200
			414R	1,810,000
			415R	2,300,000
			416R	1,900,000

association of mouse virulence with suggested possible differences in human virulence. Thus the mouse virulence of isolates from fatal cases is not significantly different from that generally found for isolates, early or late, from nonfatal cases.

Two kinds of exceptions to this generalization are apparent. First, some cases, notably 100, 130, and 410, appeared to be infected with vibrios of relatively low mouse virulence. Second, the R strains were of much reduced virulence for the mouse. R strains are defined as those agglutinating in anti-R serum; the colonial morphology of these strains was identical with that of O group I strains that did not agglutinate in anti-R serum, but in some cases the consistency of the colony was more friable. It is probably the appearance of R strains that accounts for the occasional reported occurrence of strains of *V. cholerae* of reduced virulence later in the disease.

In two series of isolates from nonfatal cases, however, there was an apparent progressive increase in mouse virulence from the first to the last isolate. The data for case 20 are shown in table 3, and those for case 170 in table 4. The isolates from case 20 were the first series to be studied, and the titrations were repeated twice. While no end is served by elaborate statistical analysis, the variability is indicated by the deviations from the averages. In the case of experiment 1, the average deviation in percent is 13.3; that in experiment 2, 40.1; and that in experiment 3, 0.1. In contrast, the difference between the average LD_{50} for strain 21 and that for strain 24 is 10.4-fold. The titration of the mouse virulence of successive isolates from case 170 was not replicated, but there the fold increase was even greater, 117 for strain 174, and almost 100 for strain 175. This progressive change in mouse virulence was not observed in other series of isolates (see table 2).

TABLE 3.—*Comparative virulence of vibrio strains isolated from case 20.*

Strain no.	LD_{50}			
	Exp. 1	Exp. 2	Exp. 3	Average
21	117,500	160,000	96,000	124,000
22	88,000	130,000	78,000	98,666
23	32,255	39,000	60,000	43,918
24	7,650	19,000	9,100	11,917

TABLE 4.—*Comparative virulence of vibrio strains isolated from case 170.*

Strain no.	LD_{50}	Fold increase
171	94,000	0
172+	17,500	5.4
173	8,000	11.8
174	800	117.0
175	950	100.0

The failure of the conventional mouse LD_{50} titration to show any apparent relation to inferred human virulence suggested that further examination of the mouse infection might be profitable. The method of quantitative blood culture described above was, therefore, applied to mouse infections with some of the vibrio strains. Representative results are shown in tables 5, 6, and 7, using strain 401 to represent the strains from fatal cases, strain 321 the strains from nonfatal cases, and 385R to represent the R strains. While the variability to be expected in this kind of experiment occurred, the tabled data represent medians rather than ex-

TABLE 5.—*Bacteremia in mice infected with strain 401.*

Hours after inoculation	Inoculum			
	16.5×10^8	16.5×10^4	16.5×10^2	16.5×10^0
2	550,000*	6,000	0	0
4	1,808,000	138,000	100	100
5	1d (1/6)†			
5.5	4d (5/6)			
5.75	1d (6/6)			
6		2,400,000	6,000	8,000
7.5		2d (2/5)		
8		3d (5/5)	510,000	476,000
9			1d (1/4)	
10			3d (4/4)	
11				2d (2/4)
11.75				2d (4/4)

* Vibrios per ml blood; ≦10.
† Deaths and accumulated deaths

TABLE 6.—*Bacteremia in mice infected with strain 321.*

Hours after inoculation	Inoculum			
	18.5×10^8	18.5×10^7	18.5×10^6	18.5×10^5
2	1,668,000*	160,500	0	0
4	10,840,000	466,000	0	0
5.75	1d (1/6)†			
6	2d (3/6	2,810,000	14,600	0
6.75	3d (6/6)	1d (1/5)		
7.5		1d (2/5)		
8		2d (4/5)	250,000	13,000
12		1d (5/5)	2,114,000	74,000
14			2d (2/4)	
20			1d (3/4)	

* Vibrios per ml blood; 0 ≤10.
† Deaths and accumulated deaths.

tremes of the differences observed (see tables 8 and 10).

It is evident from these data that strains 401 and 321, isolated from fatal and nonfatal cases respectively, differed

TABLE 7.—*Bacteremia in mice infected with strain 385R.*

Hours after inoculation	Inoculum			
	25×10^8	25×10^7	25×10^6	25×10^5
2	3,900*	0	0	0
4	19,000	180	0	0
6	789,000	20,800	2,100	0
10	1d (1/5)†	380,000	7,460	840
16	2d (3/5)	9,000	320	0

* Vibrios per ml blood; 0 ≤10.
† Deaths and accumulated deaths

from one another to a considerable degree with respect to the numbers of vibrios cultivable from the blood when deaths began to occur, and in the rates at which numbers of vibrios present in

TABLE 8.—*Summary of bacteremia in fatal mouse infections.*

Origin	Strain no.	Inoculum	Deaths	Level of bacteremia*	Fold increase in blood†
Fatal cases	141	17.3×10^3	4/4	655,000	15.2
	142	15.0×10^3	4/4	59,000	17.3
	143	16.3×10^3	3/3	1,020,000	13.8
	144	13.4×10^4	4/4	2,600	10.4
	371	13.2×10^3	4/4	360,000	9.5
	372	19.3×10^3	4/4	500,000	28.0
	401	16.5×10^3	4/4	476,000	47.0
Nonfatal cases	22	2.5×10^8	3/4	7,000,000	5.9
	24	18.0×10^3	5/5	9,760,000	5.0
	171	9.4×10^3	6/6	2,480,000	1.9
	321	18.5×10^8	5/5	2,810,000	9.9

* Vibrios per ml of blood.
† Mean fold increase per 2 hours.

TABLE 9.—*Comparison of vibrio strains from fatal and nonfatal cases.*

Criterion	Fatal	Nonfatal	Fold difference
Mean fold increase in blood per 2 hours	20.1	5.5	3.65
Mean level of bacteremia preceding death	438,892	5,512,500	12.56

the blood increased during the period of observation. The data on the R strain, 385R, are difficult to compare with the other two because of its low virulence for the mouse. Additional strains were studied to gave a total of 16 strains examined by this method. Of these, 7 had been isolated from fatal cases, 5 were O group I strains isolated from nonfatal cases, and 4 were R strains. Data on the O group I strains are summarized in table 8. The levels of bacteremia shown are those found in the last culture before deaths began to occur in the groups of animals receiving the smallest inoculum to give 100% mortality, or nearly so, on the assumption that these are reasonably comparable to one another. In the calculation of the fold increases in the numbers of cultivable vibrios per 2 hour interval, only those groups for which 3 or more successive bacterial counts were available were used, and negative culture

TABLE 10.—*Percentage of inoculated vibrios appearing in the blood at 2 hours.*

Disease	Strain no.	Approximate inoculum	
		1×10^7	1×10^6
Fatal	141	1.08	0.92
	142	5.63	6.00
	143	9.69	6.13
	144	4.24	1.49
	371	2.08	2.21
	372	1.52	10.36
	401	3.33	3.64
Nonfatal (O strains)	22	2.42	10.00
	24	4.92	5.38
	171	26.46	158.15
	175	24.32	49.05
	321	9.02	8.67
Nonfatal (R strains)	385	0.0015	0
	416b	0.0046	0.005
	416d	0.0030	0.0020
	416e	0.0011	0

was not counted. The R strains are not included in table 8 since there was no inoculation series giving 100% mortality. It is evident that there is a considerable degree of consistency within the groups of strains from fatal and nonfatal cases, that the variability within each group is approximately the same, and that the groups differ appreciably from one another

Using the mean fold increase in numbers of vibrios in the blood per 2-hour interval, and the mean level of bacteremia immediately preceding deaths as the criteria, the differences between vibrio strains of fatal and nonfatal origin are shown in table 9, with 3.65 and 12.56-fold differences respectively between the two groups. Again, the small numbers hardly justify statistical analysis though the mean fold increases in numbers of vibrios were based on 39 individual rates in the case of the strains of fatal origin, and 22 individual rates for those from nonfatal cases.

The fulminating nature of the fatal bacteremia led to attempts to pass the infection directly from mouse to mouse without the use of mucin. Mice were infected intraperitoneally with vibrios in mucin suspension, blood taken at 4 to 6 hours after inoculation, pooled and inoculated intravenously into other mice. This experiment was replicated a number of times using varied primary inoculums, but in no instance was it possible to infect mice by the intravenous inoculation of heavily infected blood. While the fold increase in numbers of vibrios in the blood was compatible with the assumption that multiplication was taking place in the circulating blood, at the same time there was no assurance that the bacteremia was more than a reflection of a generalized infection. Failure to transmit the blood infection was consistent with some such secondary role of the bacteremia.

No attempt was made to assess the rate of increase in numbers of the peritoneal cavity infection, but data on the level of bacteremia 2 hours after intraperitoneal inoculation were aggregated in the form of percentage of the inoculum cultivable from the blood at this time. These data are shown in table 10. It seems probable that at this early stage in the infection, and particularly since vibrios were detectable in the blood this early only in animals that had received relatively large inoculums, that the bacteria in the blood represented spillover from the peritoneal infection for the most part. It is apparent in these data that, with two, or possibly three, exceptions the percentages are remarkably constant for the O group I strains with no differentiation between those of fatal and nonfatal origin. From this point of view, the R strains were sharply set apart from the O group I strains with more than 100 fold differences in the observed percentages, and suggesting that their reduced mouse virulence may be due to more effective control of the intraperitoneal infection by the host, or relative inability to spread from the focus of infection.

The peritoneal infection could be passed to a second mouse using peritoneal washings clarified by high speed centrifugation but containing about 1×10^3 viable vibrios per ml. An inoculum of 0.5 ml of this material sufficed to produce a fatal infection in about half the mice inoculated, to give an LD_{50} about 2 logs greater than that observed with culture vibrios suspended in 5 percent mucin (see table 2). Control experiments in which mice were inoculated intraperitoneally with sterile mucin solution, sacrificed, and the centrifuged peritoneal washings used for suspension of serial dilutions of culture vibrios gave an LD_{50} of more

than 1×10^7 vibrios, a level closely similar to that obtainable with vibrios suspended in salt solution, and approaching the toxicity level. These results were taken to suggest either an enhanced virulence for the mouse of vibrios growing in the peritoneal cavity of that animal, or the presence of some virulence-enhancing substance other than mucin. Titration of the virulence of vibrios sedimented from the infected peritoneal washings gave no indication of increased virulence, nor was it possible to remove viable vibrios from the infected washings by filtration, heating at 56 C for 1 hour, or treatment with ethyl ether, and show that the material enhanced the virulence of culture vibrios suspended in it. Since the question of a selective enhancement of virulence for the mouse appeared to be somewhat irrelevant to a correlation of mouse virulence with postulated virulence of the vibrios for man, the suggestive observation of transmission of the peritoneal infection was not pursued further.

DISCUSSION

From the evidence presented here there seems to be no clear correlation between the mouse virulence of *V. cholerae* measured by the conventional LD_{50} technic and human virulence that might be inferred from clinical data. Thus 8 vibrio strains isolated from 4 fatal cases of the disease, 4 of the strains from one case, showed mouse LD_{50}'s ranging from 38 to 536, and 15 strains isolated from 9 nonfatal cases had substantially the same mouse virulence, ranging from 78 to 550. Seven strains (table 2) isolated from 3 nonfatal cases, however, were of less virulence for the mouse with LD_{50}'s ranging from 7,600 to 127,000; 1 case contributed 4 of these strains, and another case 2 strains. While it would appear that nonfatal cholera in man can be caused by vibrio strains of mouse virulence as great as that of strains from fatal cases, and also by strains of much less virulence for the mouse, the small numbers studied do not justify the conclusion that fatal cholera is invariably an infection with strains of high mouse virulence.

The results also offer no support for the assumption that vibrio strains of O group I isolated late in the disease are of reduced virulence for either man or mouse. In fact the contrary was observed in two series of 4 and 5 isolates in which the mouse virulence increased progressively from the first to last isolate. Since such systematic changes in mouse virulence were not observed in other, seemingly identical, series of isolates, it is difficult to account for their occurrence or attach significance to them. These observations may be taken to further substantiate the tentative conclusion that initiation of the human disease is independent of the mouse virulence of the invading microorganisms.

The R variants appearing later in the disease were of markedly reduced mouse virulence and their appearance was apparently unrelated to the mouse virulence of earlier isolates in that they were recovered from individuals infected with strains of both high and low mouse virulence.

Confidence in such implications of the data presented here is necessarily tempered by the number of transplants unavoidably intervening between primary isolation and mouse inoculation even though a constant colonial type was maintained.

The question of the relation between mouse and human virulence of *V. cholerae* is of practical significance in the selection of vaccine strains and assay of their immunogenic potency. The present practice of selecting vaccine and challenge strains on the basis of high

mouse virulence gives high immunogenic potency assays by the use of challenge strains having LD_{50}'s far below toxicity levels. It is a method of protecting the mouse against cholera, but not necessarily one of providing a preparation that will give an effective prophylactic immunity against the disease in man. The data reported here suggest that the mouse LD_{50} criterion of virulence is, in fact, quite irrelevant to cholera in man.

When the mouse infection was followed by periodic quantitative blood culture, the results suggested that it may be possible to differentiate between vibrio strains of fatal and nonfatal origin, and that the relative avirulence of R strains is different from that of less virulent O group I strains. It may be reasonably assumed that the primary focus of infection is established at the point of inoculation, the peritoneal cavity. Conceivably the infected area may function as a quiescent reservoir of infection, but it seems more probable that active multiplication occurs to give a true infection; certainly very large numbers of viable vibrios were present in peritoneal washings taken at 6 hours after inoculation when the bacteremia had already reached maximal or near maximal levels. In any case, it is clear that the infectious agent is disseminated from the peritoneal cavity, certainly into the bloodstream and possibly more widely. The immediate cause of death is unknown other than a possible endotoxin toxemia.

Viewing the results reported here against such a background, it is apparent that the rate of spillover of vibrios from the peritoneal cavity into the blood is appreciable, possibly 1 to 10% in the first 2 hours of infection in the case of O group I vibrios. The regular rate of increase of vibrios cultivable from the blood is consistent with an assumption of multiplication in the bloodstream; the maximum fold increase per 2-hour period observed, 47.0, is equivalent to a generation time of just under 23 minutes if all progeny were cultivable. It is unlikely, though, that the observed increases in numbers can be attributed to multiplication in the bloodstream alone. Undoubtedly the bacteremia continues to be fed from the peritoneal cavity at the same or possibly an increasing rate from a continuously growing population. Thus there are at least three obvious contributing factors of uncertain quantitative importance to the observed increase in numbers of vibrios in the blood. In addition, some unknown portion of new cells may be destroyed by the defense mechanisms of the normal animal. Presumably these are highly effective in the intact animal since mucin is required for the initiation of infection by other than very large numbers of vibrios, but may be overwhelmed early in the infection since there was no evidence that the rate of increase in numbers of vibrios in the blood increased as the infection progressed. In general the observed numbers may be taken to represent multiplication and/or leakage of vibrios into the bloodstream minus those destroyed or syphoned off into tissues other than blood.

Thus in assaying the indicated differences between O group I vibrios of fatal and nonfatal origin, and assuming the blood to be a representative tissue, it may be concluded that, on the whole, strains isolated from fatal cases of the disease were more invasive by a factor of 3.6 than strains isolated from nonfatal cases. In view of the considerations outlined above, it is not clear what invasive means.

The other criterion of differentiation between O group I strains of fatal and nonfatal origin is the mean level of

bacteremia reached before deaths began to occur. As shown earlier (table 9), this was found to be about 12.5-fold greater for strains of nonfatal origin. This might be taken to suggest that the strains isolated from fatal cases were more toxic in that fewer vibrios were required to produce a fatal toxemia. This appears to be unlikely for two reasons. First, differences as great as 10-fold or more should be apparent in toxicity studies. Gallut (1954) found detectable differences in the diffusible "4-hour" toxin between some of these same strains, but not as great as 10 fold nor related to fatal or nonfatal origin, and Freter (1956), who studied whole cell toxicity of these strains, found no differences in toxicity associated with origin. Second, with large inoculums, 1×10^7 or more vibrios, which are near the toxicity level and therefore should minimize the effects of tissue invasion, the average survival time was 4.65 hours, while that of animals receiving nonfatal strains was 5.83 hours. These differences do not appear to be compatible with 10-fold differences in toxicity even assuming an unusually high toxicity of vibrios produced by in vivo multiplication.

The behavior of the avirulent R strains in the mouse may have some significance in the interpretation of the observed differences between O group I strains of different origin. Comparable data were not available since the LD_{50}'s were not far below toxicity levels and 100% mortality groups in which the toxicity of the inoculum could be disregarded did not occur. Nevertheless, the mean level of bacteremia could be approximated at 5 to 8×10^7 vibrios per ml of blood, and the mean fold increase in the blood per 2-hour interval was 4.6. On the basis of these two criteria, the R strains were intermediate between vibrio strains of fatal origin and those isolated from nonfatal cases of the disease, resembling the former in mean level of bacteremia, and the latter in rate of increase in the blood. As pointed out earlier, however, they differed from both with respect to the percentage of the inoculum found in the blood at 2 hours (see table 10), and it may be inferred that they spread from the initial peritoneal cavity infection much less readily than the O group I strains.

Assuming that all the strains of *V. cholerae* studied here are equally toxic, equally able to set up an initial infection in the presence of mucin, and capable of substantially the same rate of uninhibited multiplication, it is possible to separate them into three groups on the basis of the effects of relative invasiveness though without definition of the causal elements of that invasiveness. On the basis of the experimental data and inferences from those data, the group of strains of predominantly fatal origin, the group of O group I strains of predominantly nonfatal origin, and the group of R strains may be characterized as follows:

(1) The O group I vibrios spread rapidly from the peritoneal cavity, with 1 to 10% of the inoculum demonstrable by blood culture at 2 hours.

(a) The strains of predominantly fatal origin increase rapidly in the blood, possibly as a consequence of an increasing rate of dissemination from the peritoneal focus of infection, multiplication in the blood stream, or failure of defense mechanisms to remove them, or any combination of these factors. The maximal level of bacteremia reached just before deaths begin to occur remains moderate, possibly because of invasion of other tissues from the hematogenous source of infection. The lethal amount of toxicity is probably widely distributed in susceptible tissues.

(b) The strains of predominantly nonfatal origin increase in numbers in

the blood at only a moderate rate, possibly because the rate of spillover from the peritoneal cavity does not increase as the infection progresses or because multiplication in the blood is not significant. It seems unlikely that the microorganisms are destroyed to any large extent in the blood because of their accumulation to high levels. For the same reason invasion of other tissues from the blood may be limited though sufficient effectively to inhibit defense mechanisms. The lethal amount of toxicity accumulated may be regarded as less widely, though reasonably effectively, distributed in susceptible tissues; this is consistent with the slightly longer survival time.

(2) The R strains appear to be limited in their ability to spread from the focus of infection in the peritoneal cavity, and reach only moderate numbers in the blood and then decline. The decrease in the level of bacteremia is consistent with the assumption that they are unable to invade other tissues to the extent of producing injury sufficient to affect the normal antibacterial and clearing mechanisms. The accumulated toxicity may not be as widely distributed in susceptible tissues but tends to be confined to the initial focus of infection, and thus contribute to irregular deaths and a prolonged survival time.

If fatality in the human infection may be taken as an indicator of the relative virulence of *V. cholerae* for man, the data reported here suggest that such virulent strains of the microorganism are demonstrably more invasive in the mouse. There is no a priori reason for such an association since Asiatic cholera is an extreme example of a true enteric infection, one in which there is little or no invasion of the tissues from the focus of infection in the lumen of the bowel. The greater invasiveness of vibrio strains of fatal origin is perhaps no more than an association with, or a reflection of, other elements of virulence of the microorganism more nearly related to the pathogenesis of the disease in man.

SUMMARY

A thionin-glycerol agar is described which allows the differentiation of widely different colonial types of *Vibrio cholerae* when examined by oblique transmitted light. Of the four main types and a variety of intermediate types, only one, designated the granular chromatic type, was found on primary isolation plates.

A total of 47 serial isolates of *V. cholerae* from fatal and nonfatal cases of the disease was examined in the 6th transplant from the original isolation plates for mouse virulence by the conventional LD_{50} method. No correlation between mouse virulence and origin of O group I strains was found, and it was concluded that human cholera may be caused by vibrio strains of either high or low mouse virulence. R strains, isolated not earlier than the 3rd day of the disease in nonfatal cases, were found to be of low mouse virulence.

A method of periodic quantitative blood culture was devised which made it possible to follow the development of the mouse infection. Infection with O group I strains rapidly disseminated from the peritoneal cavity infection, with 1 to 10% of the inoculum cultivable from the blood at 2 hours, while R strains showed less than 0.005% of the inoculum present in the blood in the same time. It was found that the mean fold increase in numbers of O group I strains of *V. cholerae* cultivable from the blood was 20.1 for strains isolated from fatal cases of the disease in contrast to 5.5 for strains isolated from nonfatal cases. Contrariwise, the mean level of bacteremia reached just before deaths began to occur was approximately

438,900 for strains of fatal origin, and 5,512,500 for strains of nonfatal origin. The data are taken to suggest that vibrio strains isolated from fatal cases of the human disease are more invasive in the mouse than those isolated from nonfatal cases.

REFERENCES

Burrows, W., Elliott, M. E. and Havens, I. 1947, Studies on immunity to Asiatic cholera. IV. The excretion of coproantibody in experimental enteric cholera in the guinea pig. J. Infect. Dis. **81**: 261–281.

Burrows, W., Mather, A. N., Elliott, M. E. and Havens, I. 1947, Studies on immunity to Asiatic cholera. III. The mouse protection test. J. Infect. Dis. **81**: 157–174.

Cantacuzene, J. and Marie, A. 1914, Cholera gastrointestinal experimental chez la cobaye. Compt. rend. Soc. de biol. **76**: 307–310.

Doorenbos, W. 1938, Vibrio problems in epidemic and endemic cholera. J. Egyptian Med. Assoc. **21**: 279–284.

Dutta, N. K. and Habbu, M. K. 1955, Experimental cholera in infant rabbits: a method for chemotherapeutic investigation. Brit. J. Pharmacol. & Chemotherapy, **10**: 153–159.

Fishel, C. W. 1955, Studies on the histamine sensitivity induced in mice by the inoculation of *Hemophilus pertussis*. Dissertation, University of Chicago.

Freter, R. 1955a, The serologic character of cholera vibrio mucinase. J. Infect. Dis. **97**: 238–245.

———. 1955b, The fatal enteric cholera infection in the guinea pig achieved by inhibition of normal enteric flora. J. Infect. Dis. **97**: 57–65.

———. 1956, Two different toxic fractions extracted from *Vibrio cholerae*. J. Infect. Dis. In press.

Gallut, J. 1954, A l'etude de la toxine cholerique: variation du pouvoir toxique de *Vibrio cholerae* (Ogawa) au cours de la maladie. Ann. Inst. Pasteur, **86**: 561.

Griffitts, J. J. 1942, The use of mucin in experimental infections of mice with *Vibrio cholerae*. Pub. Health Rep. **57**: 707–710.

Henry, B. S. 1933, Dissociation in the genus Brucella. J. Infect. Dis. **52**: 374–402.

de Moor, C. E. 1938, An El Tor vibrio as a cause of a cholera epidemic in Celebes. Bull. Office Internat. d'Hyg. Publique, **30**: 1510–1519.

Pandit, C. G. and Hora, S. L. 1951, The probable role of the hilsa fish, *Hilsa ilisha* (Ham) in maintaining cholera endemicity in India. Indian J. Med. Sc. **5**: 343–356.

Pittman, M. 1951, Sensitivity of mice to histamine during respiratory infection by *Hemophilus pertusssis*. Proc. Soc. Exptl. Biol. & Med. **77**: 70–74.

Ranta, L. E. and Dolman, C. E. 1943, Observations on cholera vaccine. Canadian J. Pub. Health, **34**: 26–37.

Sanarelli, G. 1921, De la pathogenie du cholera V. le "cholera intestinal" des jeunes animaux. Ann. Inst. Pasteur, **35**: 745–796.

Siler, J. F. et al. 1941, Immunization to typhoid fever. Am. J. Hyg. Monograph no. 17.

Swaroop, S. and Pollitzer, R. 1955, Cholera studies. 2. World incidence. Bull. W. H. O., **12**: 311–358.

FURTHER EXPERIMENTS ON THE SELECTIVE SUSCEPTIBILITY OF SPIDER MONKEYS TO POLIOMYELITIC INFECTION

CLAUS W. JUNGEBLUT AND GONZALO BAUTISTA, JR.

From the Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York City

It is generally believed that Old World monkeys are more susceptible to infection with poliomyelitis virus than those of the Western hemisphere. Among the New World monkeys two South American species of spider monkeys (*Ateles ater, Ateles fusciceps robustus*) were reported as being naturally refractory to poliomyelitis virus by MacKay and Schroeder[1] in 1935 and again in 1952 by Rodaniche.[2] On the other hand, Gomez,[3] working with Mexican spider monkeys (*Ateles neglectus*) in 1951, had no difficulty in producing paralytic infection in these animals. The aforementioned workers used for their experiments the Flexner MV strain (mixed virus) or freshly isolated strains which had not been typed serologically.

It seemed possible that the divergent results were influenced less by the selection of monkey than by the choice of strain. Therefore, the status of susceptibility of spider monkeys from South or Central America to poliomyelitic infection was reinvestigated in 1954 by Jungeblut and Rodaniche.[4] All three serological types of virus were employed in this work as well as Col SK virus, since the latter virus shares many of the properties of poliomyelitis virus. It was found that spider monkeys (*Ateles fusciceps robustus, Ateles geoffreyi*) were highly susceptible to intracerebral infection with three strains of type I poliomyelitis virus (Brunhilde, Mahoney, J.V.) and Col SK virus but were completely refractory to infection by the same route with two strains each of type II (Aycock, MEF) and type III (Leon, Farabaugh) poliomyelitis virus. Moreover, asymptomatic infection with type II or type III virus seemed to confer various degrees of protection against reinfection with type I virus (Brunhilde). Paralysis could not be produced in spider monkeys by feeding type I or Col SK virus. The susceptibility of spider monkeys to type I poliomyelitis virus is confirmed by the results obtained by Pizarro and Ramirez[5] with Mexican spider monkeys (*Ateles vellerosus*). These observations were published independently and almost simultaneously in 1954.

The present paper presents a series of experiments designed to further study the mechanism of this selective susceptibility.

Received for publication May 7, 1956.

Presented in part before the Society of Experimental Pathology at the Federation meetings in San Francisco, April 1955.

Aided by a grant from the Sister Elizabeth Kenny Foundation.

1. MacKay, E. M. and Schroeder, C. R. 1935, Resistance of spider monkey (*Ateles ater*) to infection with virus of acute anterior poliomyelitis. Proc. Soc. Exper. Biol. & Med. **33**: 373–374.
2. de Rodaniche, E. C. 1952, Susceptibility of certain species of panamanian monkeys to the virus of acute anterior poliomyelitis, Am. J. Trop. Med. & Hyg. **1:2**: 205–209.
3. Gomez, J. 1951, Algunos aspectos de la poliomyelitis experimental, Thesis, University of Puebla, Puebla, Pue., Mexico 1–67.
4. Jungeblut, C. W. and de Rodaniche, E. C. 1954, Selective susceptibility of Ateles monkeys to infection with Type I poliomyelitis and Col SK virus. Proc. Soc. Exper. Biol. & Med. **86**: 604–606.
5. Pizarro, E. and Ramirez, M. 1954, Transmisión experimental de la poliomielitis a Ateles vellerosus (mono araña), Ciencia (Mexico). **14**: 15–17.

EXPERIMENTAL WORK

Material and methods.—Spider monkeys from Mexico, Panama and Colombia were used in this work; they were young animals weighing about 1 to 1.5 kg. The strains of virus employed were: poliomyelitis type I (Brunhilde), poliomyelitis type II (Aycock), poliomyelitis type III (Leon) and Col SK virus. The three stains of poliomyelitis virus were available as frozen rhesus passage cords; the ID_{50} end point of infectivity of these strains, as titrated in rhesus monkeys, was between 10^{-6} and 10^{-7}. The source of Col SK virus was brain harvested from paralyzed mice; the ID_{50} of the virus in mice was 10^{-9}.

Experiment I.—This experiment deals with attempts to break down the resistance of spider monkeys to infection with type II or type III poliomyelitis virus by the administration of corticosteroids. A group of 6 spider monkeys were given daily injections of 10 mg cortisone for 5 successive days. Three monkeys were then inoculated intracerebrally with 1 cc of a 10^{-1} suspension of type II virus, the other 3 with the same dose of type III virus. None of the animals in this group showed any paralytic symptoms, whereas 4 accompanying rhesus control monkeys (2 with type II, 2 with type III virus) all became paralyzed.

Experiment II.—As a next step, the possibility was considered that spider monkeys may fail to develop paralysis with type II or type III poliomyelitis virus because of their inability to form spinal variants from the intracerebrally injected virus. Therefore, 4 spider monkeys were infected with either type II or type III virus by placing the inoculum directly into the lumbar cord. All 4 monkeys survived without any paralytic symptoms.

Experiment III.—Inasmuch as the negative results of the first two experiments provided no answer to our problem, it was decided to trace the fate of nonpathogenic type III poliomyelitis virus in neural and extraneural tissues of spider monkeys following intracerebral infection. For purpose of control, similar tests were carried out with spider monkeys infected with the pathogenic type I virus. In these experiments spiders were sacrificed at various intervals after infection and the injected brain site, the cord, the blood and the spleen were tested for the presence of active virus by intracerebral transfer of the ground tissue suspensions to rhesus monkeys. The results are given in table 1. As will be seen, active type III virus could regularly be recovered from the injected brain sites up to 96 hours, but not from the cord or extraneural tissues. Because of the strict confinement of the virus to the site of injection, it is not surprising that specific antibodies could not be demonstrated in the serum of asymptomatic type III-infected spiders, at early or late intervals. By contrast, it proved impossible to recover free type I virus from the brain sites of infected spider monkeys up to the 4th day. Active virus, however, could then be demonstrated, begining with the 5th day, from both brain and cord when some infected spiders al-

TABLE 1.—*Recovery of nonpathogenic (Leon) and of pathogenic (Brunhilde) virus from infected spider monkeys.*

Virus strain	Interval, days	Virus content			
		Brain site	Cord	Blood	Spleen
Leon (type III)	1	+	–	–	
	2	+			
	3	+	–	–	–
	4	+			
	5	–	–	–	–
	7	–	–		
Brunhilde (type I)	1	–	–	–	–
	2	–			
	3	–	–	–	–
	4	–			
	5	+	+		
	7	+	+		

+, virus recovered.
–, no virus recovered.

ready showed initial symptoms of paralysis.

Comparing the two sets of observations, one is led to the conclusion that in the case of the susceptible virus-host combination the virus is tightly bound by cell receptors during the entire incubation period and becomes released only at the symptomatic stage. A similar lag phase was found in earlier experiments on the survival of type II virus (Aycock strain) at the brain site of intracerebrally infected rhesus monkeys.[6] On the other hand, in the case of the insusceptible virus-host combination the virus apparently remains as free as if it were deposited in a test tube for 4 days and then completely disappears from the tissue. It seemed possible that the virus, at that time, may be converted into a nonpathogenic but immunizing agent. However, when rhesus monkeys were immunized with avirulent Leon spider brain sites, they failed to develop challenge resistance and no neutralizing antibodies could be detected in the serum.

Experiment IV.—The following experiment is concerned with further studies on the cross protection that asymptomatic type III-infected spider monkeys may acquire against intracerebral reinfection with the pathogenic type I virus. For this purpose groups of spider monkeys were injected intracerebrally with 0.5 cc of 10^{-1} type III virus and, at various intervals thereafter, were reinfected by the same route with 0.5 cc of 10^{-1} type I virus. The results are brought together in table 2. It will be seen that 3 of 4 spiders reinfected between the 1st and 4th day survived without symptoms, whereas all of 5 spiders reinfected between the 5th and 25th day developed paralysis. The protection at the early interval coincides with the actual persistence of live type III virus in the brain; it is therefore perhaps best accounted for by in vivo interference between the two virus strains.

TABLE 2.—*Resistance of asymptomatic Leon-infected spider monkeys to reinfection with Brunhilde virus.*

Interval, days	Number of monkeys	Result of Brunhilde reinfection (intracerebral)	
		Paralysis	No paralysis
1–4	4	1 (19 days)	3
5–25	5	5 (8–10 days)	0
Brunhilde controls	4	4 (8–11 days)	0

Experiment V.—In view of the results above, some experiments were finally run on in vitro interference between the several virus strains. In this work advantage was taken of the fact that the two species of monkeys rhesus and spider, possessed differential susceptibility for the three types of poliomyelitis virus and Col SK virus. In other words, whereas spider monkeys are paralyzed only by type I poliomyelitis virus and by Col SK virus, rhesus monkeys are evenly susceptible to all three types of poliomyelitis virus but resist infection with Col SK virus. Accordingly, mixtures consisting of equal parts of the pathogenic and nonpathogenic strains were prepared in vitro. Within minutes after mixing, the combinations were then injected intracerebrally, in 1 cc volume, into groups of spider and rhesus monkeys. Appropriate controls with single virus infection accompanied these tests. The results are given in table 3. It will be seen that of the two spider-pathogenic viruses, type I poliomyelitis virus was effectively interfered with by type III but not by type II poliomyelitis virus, whereas Col SK virus could not be suppressed by either type II or type III

6. Jungeblut, C. W. 1931, Fate of poliomyelitis virus in the brain after intracerebral inoculation of normal and convalescent monkeys. Proc. Soc. Exper. Biol. & Med. **28**: 1093–1096.

TABLE 3.—*Interference tests in spider and in rhesus monkeys.*

Pathogenic strain	Nonpathogenic strain	Infection with virus mixture	Controls	
		In spider monkeys		
Brunhilde type I	Aycock Type II	3/3	Brunhilde	7/8
Brunhilde type I	Leon Type III	0/10	Col SK	3/3
			Aycock	0/6
Col SK	Aycock Type II	6/6	Leon	0/7
Col SK	Leon Type III	6/6		
		In rhesus monkeys		
Brunhilde type I	Col SK	0/2	Brunhilde	2/2
Aycock type II	Col SK	0/2	Aycock	2/2
Leon type III	Col SK	4/4	Leon	4/4
			Col SK	0/2

Numerator: monkeys paralyzed.
Denominator: monkeys injected.

poliomyelitis virus. On the other hand, of the three rhesus-pathogenic strains of poliomyelitis virus, type I and type II virus were completely suppressed by Col SK virus whereas type III virus remained unaffected.

The data offer confirmatory evidence of a definite interference system in spiders between type I and type III poliomyelitis virus, which had already been suggested by experiment IV. They also demonstrate unilateral interference effects in rhesus monkeys between Col SK virus and type I and type II poliomyelitis virus. That reverse interference between Col SK and poliomyelitis virus could not be demonstrated in spiders may be due to the higher virulence of Col SK virus as compared with the several poliomyelitis viruses. However, no final conclusion is possible on this point since our experiments were not run on a quantitative scale.

DISCUSSION

The observations reported in this paper are of interest because they demonstrate the existence in Ateles monkeys of a highly selective, nonimmunological mechanism of resistance against type II and type III poliomyelitis virus. The data also offer evidence to suggest that this natural resistance may be connected with the absence of type-specific cell receptors in the central nervous system. Our findings are in essential agreement with results obtained by Kaplan[7] with another New World monkey, *Cebus capucina*; this species is considered as naturally refractory to poliomyelitis passage virus, but may be susceptible to infection with human fecal virus.[8–11] Thus, tissue cultures prepared from capuchin kidneys not only failed to support prototype virus multiplication (type I—Brunhilde; type II—Y SK; type III—Leon) but the cells also failed to adsorb measurable quantities of virus. Interestingly enough, when human fecal virus was used, instead of passage virus, type I virus persisted in the tissue culture tubes—with little or no cytopathogenicity—but not type II or type III virus. The sum total of these observations, all of which are concerned with the selective response of a given tissue to the three serological types of poliomyelitis virus, assumes additional significance in view of recent work by Silverberg, Habel and Shelokov,[12] which reveals marked differences

7. Kaplan, A. S. 1955, Comparison of susceptible and resistant cells to infection with poliomyelitis virus. Ann. N. Y. Acad. Sc. **61**: 830–839.
8. Flexner, S. and Lewis, P. A. 1910, Experimental epidemic poliomyelitis in monkeys. J. Exper. Med. **12**: 227–255.
9. Kraus, R. and Kantor, L. 1917, Rev. d. Inst. bact., Santiago de Chile, **1**: 43–45.
10. Jungeblut, C. W. and Engle, E. T. 1932, On the property of certain normal animal sera to neutralize the virus of poliomyelitis. Proc. Soc. Exper. Biol. & Med. **29**: 879–883.
11. Melnick, J. L. and Paul, J. R. 1943, Susceptibility of *Cebus capucina* (South American ringtail monkey) and *Cercopithecus cephus* (African mustache monkey) to poliomyelitis virus. J. Exper. Med. **78**: 273–283.
12. Silverberg, R. J., Habel, K. and Shelokov, A. 1955, Relative pathogenicity of newly isolated strains of poliomyelitis for monkey kidney cells and mice. Proc. Soc. Exper. Biol. & Med. **90**: 655–658.

in the adaptability to mice of freshly isolated strains of type I, II and III virus.

The reinfection and interference experiments throw new light on the ability of type III poliomyelitis virus to suppress multiplication of type I virus in a suitable tissue environment. Our data show that such antagonistic effects in spider monkeys can be elicited equally well when the interfering viruses are injected seperately, albeit within certain time limits, as with in vitro prepared mixtures. A broad reciprocal interference between all three types of poliomyelitis virus, depending on time and dosage, has been reported by Ledinko and Melnick in tissue cultures of monkey testes.[13] As far as the interference between Col SK virus and the several types of poliomyelitis virus in rhesus monkeys is concerned, our results are in harmony with earlier observations on the suppression of type II virus by Col SK virus;[14–18] suppression of type I virus, however, has hitherto not been realized.

SUMMARY AND CONCLUSIONS

1. The selective susceptibility of Spider monkeys (*Ateles niger*) from various parts of Central or South America to type I poliomyelitis virus is confirmed. The nonimmunological resistance of these animals to infection with type II and type III poliomyelitis virus could not be broken down by intraspinal injection of the virus or by cortisone administration.

2. Type III poliomyelitis virus was readily recoverable in active form from the brain sites of intracerebrally infected spider monkeys up to the 4th day after inoculation. By contrast, type I poliomyelitis virus could not be recovered during this interval under similar experimental conditions. This observation suggests that the natural resistance of the spider monkey to type III virus infection is connected with the absence of type-specific cell receptors.

3. Spider monkeys following asymptomatic infection with type III poliomyelitis virus may show a certain degree of resistance when challenged with type I virus. The protective effect appears to depend on survival of active type III virus at the site of injection and not on antibody action.

4. In vitro prepared mixtures of the pathogenic type I and the nonpathogenic type III virus, when injected intracerebrally into spider monkeys, failed to produce paralysis. This interference was not observed with mixtures consisting of type I and type II virus.

13. Ledinko, N. and Melnick, J. L. 1954, Interference between poliomyelitis viruses in tissue culture. J. Exper. Med. **100**: 247–267.
14. Jungeblut, C. W. and Sanders, M. 1940 Studies of a murine strain of poliomyelitis virus in cotton rats and white mice. J. Exper. Med. **72**: 407–436.
15. Jungeblut, C. W. and Sanders, M. 1942, Studies in rodent poliomyelitis. I. Further experiments with the murine strain of SK poliomyelitis virus. J. Exper. Med. **75**: 611–629.
16. Jungeblut, C. W. and Sanders, M. 1942, Studies in rodent poliomyelitis. V. Interference between murine and monkey poliomyelitis virus. J. Exper. Med. **76**: 127–142.
17. Jungeblut, C. W. 1945, Studies in rodent poliomyelitis. VI. Further observations on interference between murine and simian strains of poliomyelitis virus. J. Exper. Med. **81**: 275–294.
18. Verlinde, J. D. and Molron, J. M. 1954 Mixed infection with type II poliomyelitis virus and Columbia SK group virus in man. Ant. v. Leeuwenhoek, **20**: 129–140.

VOLUME 99 JULY-AUGUST 1956 NUMBER 1

The Journal of Infectious Diseases

TABLE OF CONTENTS

PAGE

ROBERT M. LEWERT AND CHANG-LING LEE. Quantitative studies of the collagenolytic enzymes of cercariae of *Schistosoma mansoni* and the larvae of *Strongyloides ratti* 1

CHANG-LING LEE AND ROBERT M. LEWERT. The maintenance of *Schistosoma mansoni* in the laboratory 15

WILLIAM H. TALIAFERRO AND DAVID W. TALMAGE. Antibodies in the rabbit with different rates of metabolic decay 21

GORDON STAGG, WILLIAM S. TANNER, AND JOHN LAVENDER. Experimental infections of native animals with *Pasteurella tularensis* 34

FRANCIS W. HUGHES AND ARTHUR L. TATUM. Effects of hypoxia and intercurrent infections on infections by *Plasmodium berghei* in rats 38

CHARLES W. FISHER. Host-parasite relationship in experimental pertussis. I. Histamine-sensitizing and protective activities of pertussis vaccine in mice 44

G. BRIGGS PHILLIPS, GROVER C. BROADWATER, MORTON KRITMAN, AND ROBERT L. ALG. Cross infections among brucella infected guinea pigs 56

LEIF M. REINGEN AND WILLIAM OKAZAKI. The susceptibility of several different laboratory animals to infection with *Leptospira pomona* 60

JOHN E. LARSH, JR., GEORGE J. RACE, AND WILLIAM B. JEFFRIES. The association in young mice of intestinal inflammation and the loss of adult worms following an initial infection with *Trichinella spiralis* 69

SUYEMOTO, W., C. M. WHEELER, D. C. CAVANAUGH, YOKO YAMAZAWA, AND TOSHIKO SHIMADA. Studies on *Pasteurella pestis* in various flea species. III. Transmission of avirulent strains of *Past. pestis* by *Xenopsylla cheopis* 72

FLORENCE B. SEIBERT. The significance of antigen-antibody reactions in tuberculosis 76

ROBERT A. HARDER, ROBERT W. QUINN, AND ROY C. AVERY. A survey of hemolytic streptococci from patients with rheumatic fever, glomerulonephritis, pharyngitis and the carrier state 84

SYED S. HUSAIN AND WILLIAM BURROWS. Studies on immunity to Asiatic cholera. VIII. The virulence of strains of *Vibrio cholerae* for the mouse 90

CLAUDE W. JUNGHERR AND CONRAD A. BAUMEISTER, JR. Further experiments on the selective susceptibility of spider monkeys to poliomyelitic infection 102